

Defective CD4⁺ T Cell Activation in the Presence of a Metalloproteinase Inhibitor

Nigel Denning Leonard Savage

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Edinburgh University Medical School



ABSTRACT

The work described in this thesis investigated the contribution of Zn²⁺ metalloprotein enzymes to human and murine CD4⁺ T cell activation. The experiments described here reveal that addition of a hydroxamic acid containing pseudopeptide, BB-3103, capable of inhibiting a broad range of metalloproteinases, to selected T cell proliferation assays, could reduce the T cell responses by up to 40% compared to controls at certain time points. B cell proliferation was not affected suggesting the metalloproteinase activity on lymphocytes is targeted specifically to CD4⁺ T cells. Phenotypic analysis of activated CD4⁺ T cells revealed that two markers were differentially expressed in the presence of BB-3103, CD27 (a co-stimulatory molecule of the TNFR family highly expressed on CD4⁺ T cells) and CD62L (L-selectin, an adhesion molecule mediating interaction between CD4⁺ T cells and endothelium). These two molecules were not shed from the surface of the cell upon activation in the BB-3103 treated samples.

Enriched CD4⁺ T cells isolated from CD62L gene deficient mice were not inhibited by the presence of BB-3103 and demonstrated deficient or delayed proliferation as compared to wild type controls even in the absence of the inhibitor at certain time points. These data suggested that expression of CD62L on the CD4⁺ T cells was necessary for complete activation. Cross-linking CD62L with monoclonal antibodies in a proliferation assay increased wild type CD4⁺ T cell responses and protected the CD4⁺ lymphocytes from BB-3103 mediated inhibition of proliferation previously observed. CD4⁺ T cells secreted reduced amounts of IL-2 in the presence of BB-3103, correlating to the results obtained from CD4⁺ T cell proliferation assays.

Signalling pathways of activated CD4⁺ T cells were investigated. Tyrosine phosphorylation of CD3 ζ ITAM sequences and ZAP-70, two key signalling molecules was found to be downregulated in the presence of BB-3103 although the method used could not accurately and quantitatively assess these downregulations. Calcium ion mobilisation, however, was

upregulated in the presence of BB-3103 which suggested that CD62L shedding plays an important role in regulating CD4⁺ T cell activation pathways.

The effect of BB-3103 was also investigated on Th₁ and Th₂ type CD4⁺ T cell proliferation. The results of these experiments were unexpected in that, although, BB-3103 was still capable of inhibiting proliferation of both these subsets when added at time 0hrs, later time point addition of the inhibitor (24 or 48hrs), increased the Th₁ type CD4⁺ T cell proliferation. The expression of CD62L on activated CD4⁺ Th₁ and Th₂ cells was subsequently investigated and, contrary to previous studies, was found to be maintained on the Th₁ population. This proposed a dual effect of CD62L on the surface of CD4⁺ T cells; since inhibition of CD62L downregulation upon activation of CD4⁺ T cells could inhibit proliferation whereas inhibition of CD62L shedding when CD4⁺ T cells were actively dividing had either no effect or increased cell proliferation.

These studies suggest that CD62L expressed on CD4⁺ T cells should not exclusively be studied as an adhesion molecule mediating interactions with endothelium, but also as a co-stimulatory molecule capable of regulating CD4⁺ T cell activation.

DECLARATION

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous application for candidature for a higher degree. All work presented in this thesis, was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

Nigel D.L. Savage

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DEDICATION

For my Parents

and

Grandparents

CONTENTS

ABSTRACT.....	2
DECLARATION	4
ACKNOWLEDGEMENTS.....	5
DEDICATION	6
CONTENTS.....	7
LIST OF TABLES	13
LIST OF FIGURES.....	14
ABBREVIATIONS.....	18
INTRODUCTION	
1.1 AIMS OF THESIS	20
1.2 INTRODUCTION LAYOUT	20
1.3 ZINC METALLOPROTEINASES.....	21
1.3.1 MATRIXINS / MATRIX METALLOPROTEINASES (MMPs).....	23
1.3.1.1 MMPs SECRETED BY LYMPHOCYTES.....	26
1.3.1.2 REGULATION OF MMPs.....	28
1.3.2 ADAMALYSINS.....	30
1.3.2.1 ADAMS SECRETED BY T LYMPHOCYTES.....	32
1.3.2.2 REGULATION OF ADAMS	33
1.3.3 DESIGN OF METALLOPROTEINASE INHIBITORS.....	33
1.4 SUBSTRATE CANDIDATES FOR METALLOPROTEINASES EXPRESSED BY LYMPHOCYTES	37
1.4.1 TNF α AND TNF RECEPTORS 1 AND 2	37
1.4.2 FasL (CD95L/APO1-L).....	39
1.4.3 IL-6 RECEPTOR COMPLEX (IL-6R)	42
1.4.4 L-SELECTIN (CD62L).....	43
1.4.5 CD43/LEUKOSIALIN.....	45
1.4.6 CD30 AND CD27	46
1.5 MECHANISMS AND REQUIREMENTS FOR CD4 ⁺ T CELL ACTIVATION.....	49
1.5.1 TCR COMPLEX: STRUCTURE AND KINETICS.....	50

1.6 TCR SIGNALLING PATHWAYS	52
1.6.1 SRC KINASES AND p56 ^{Lck} ACTIVATION	52
1.6.2 IMPORTANCE OF CD3 ζ ITAM PHOSPHORYLATION	54
1.6.3 RECRUITMENT AND ACTIVATION OF ZAP-70	55
1.6.4 ADAPTER MOLECULES	56
1.6.4.1 LAT (LINKER FOR ACTIVATION OF T CELLS).....	56
1.6.4.2 SLP-76.....	56
1.6.5 PHOSPHOLIPASE C γ 1 (PLC γ 1) AND DOWNSTREAM EVENTS	57
1.6.6 CALCIUM	57
1.6.7 RAS ACTIVATION	58
1.7 CD4⁺ T LYMPHOCYTE SUBSETS- TH₁ VS TH₂	60
1.7.1 ANTIGEN DOSE	60
1.7.2 COSTIMULATION	61
1.7.3 CYTOKINE ENVIRONMENT	63
MATERIALS AND METHODS	
2.1 CELL ISOLATIONS AND PURIFICATIONS	65
2.1.1 MURINE SPLEEN	65
2.1.2 HUMAN PBMC ISOLATION	65
2.1.3 CELL PURIFICATIONS	66
2.2 IMMUNOLOGICAL ASSAYS	67
2.2.1 MITOGEN PROLIFERATION	67
2.2.2 POLYCLONAL TCR STIMULATION USING PLATE BOUND AND SOLUBLE ANTIBODIES	68
2.2.3 CELL COUNTS	69
2.2.4 APOPTOSIS ASSAY (ANNEXIN-V-FITC STAINING)	69
2.2.5 FLOW CYTOMETRY	69
2.2.6 ENZYME LINKED IMMUNOSORBANT ASSAYS (ELISAs).....	70
2.3 SIGNALLING	71
2.3.1 IMMUNOPRECIPITATION.....	71
2.3.2 IMMUNOBLOTTING	72
2.3.3 CALCIUM DETECTION IN HUMAN CD4 ⁺ T CELLS	73
2.4 CD4⁺ T CELLS SUBSETS.....	74
2.4.1 HUMAN CD4 ⁺ T CELL CLONE PROLIFERATION.....	74
2.4.2 PROLIFERATION OF PBMCs FROM ATOPIC AND NON-ATOPIC VOLUNTEERS	75

2.4.3 GENERATION OF MURINE Th ₁ AND Th ₂ TYPE CELLS.....	75
2.5 MATERIALS AND BUFFERS	76
RESULTS AND DISCUSSIONS	
CHAPTER 3	
3.1 PMA/IONOMYCIN INDUCED PROLIFERATION IS UNAFFECTED BY BB-3103.....	79
3.2 BB-3103 DOES NOT AFFECT B LYMPHOCYTE PROLIFERATION	81
3.2.1 BB-3103 DOES NOT AFFECT LPS SPLENOCYTE PROLIFERATION.....	81
3.2.2 BB-3103 DOES NOT MODULATE BCR MEDIATED B CELL PROLIFERATION	82
3.3 MITOGEN DRIVEN T LYMPHOCYTE PROLIFERATION IS DOWNREGULATED BY BB-3103	83
3.3.1 BB-3103 AFFECTS ConA MEDIATED T CELL PROLIFERATION	83
3.3.2 BB-3103 REDUCES PWM MEDIATED T CELL PROLIFERATION	84
3.4 T LYMPHOCYTE PROLIFERATION IS AFFECTED BY BB-3103 IN THE ABSENCE OF ACCESSORY CELLS	85
3.4.1 BB-3103 INHIBITS THE PROLIFERATION OF PURIFIED CD90 ⁺ CELLS	86
3.4.2 BB-3103 INHIBITS THE PROLIFERATION OF PURIFIED CD4 ⁺ CELLS	87
3.4.3 BB-3103 INHIBITS THE PROLIFERATION OF PURIFIED CD8 ⁺ CELLS	88
DISCUSSION (CHAPTER 3)	89
CHAPTER 4	
4. THE INHIBITION OF T LYMPHOCYTE PROLIFERATION IS NOT DUE TO APOPTOSIS OR TOXICITY	92
4.1 BB-3103 INHIBITS EARLY CD4 ⁺ T CELL EXPANSION.....	93
4.2 PRESENCE OF BB-3103 DOES NOT INCREASE APOPTOSIS	94
4.3 BB-3103 DOES NOT AFFECT IL-2 DEPENDENT AND INDEPENDENT TRANSFORMED T CELL LINES	96
4.4 IC ₅₀ VALUES FOR BB-3103 IN T CELL PROLIFERATION.....	99
DISCUSSION (CHAPTER 4)	100
CHAPTER 5	
5.1 BB-3103 REDUCES IL-2 SECRETION IN ACTIVATED CD4 ⁺ T CELLS.....	103
5.2 PHENOTYPIC ANALYSIS OF CD4 ⁺ T CELLS IN THE PRESENCE OF BB-3103	104
5.2.1 BB-3103 AFFECTS CD27 EXPRESSION ON CD4 ⁺ T CELLS	105

5.2.2	CD62L EXPRESSION ON CD4 ⁺ T CELLS IS MAINTAINED IN THE PRESENCE OF BB-3103	107
5.2.3	BB-3103 DOES NOT AFFECT CD25 AND CD69 EXPRESSION ON CD4 ⁺ T CELLS	109
5.2.4	BB-2116 AFFECTS TNF α AND TNFRII RELEASE FROM HUMAN PBMCs *	111
5.2.4.1	EXOGENOUS TNF α DOES NOT RECONSTITUTE PBMC PROLIFERATION*	112
DISCUSSION (CHAPTER 5)		113
CHAPTER 6		
6.1	PROLIFERATION OF CD4 ⁺ T CELLS FROM CD62L GENE DEFICIENT ANIMALS IS NOT INHIBITED BY BB-3103	118
6.2	CYTOKINE SECRETION FROM CD62L KO CD4 ⁺ T CELLS IS NOT AFFECTED BY BB-3103	119
6.3	PHENOTYPIC ANALYSIS OF CD62L DEFICIENT CD4 ⁺ T CELLS	120
6.3.1	PHENOTYPIC ANALYSIS OF CD62L KO CD4 ⁺ T CELLS IN THE PRESENCE OF BB-3103	121
6.3.1	BB-3103 DOES NOT MODULATE CD25 EXPRESSION ON CD62L KO CD4 ⁺ T CELLS	122
6.3.2	BB-3103 DOES NOT REDUCE CD69 EXPRESSION ON CD62L KO CD4 ⁺ T CELLS	123
6.3.3	BB-3103 AFFECTS CD27 EXPRESSION ON CD62L KO CD4 ⁺ T CELLS	125
6.4	SOLUBLE ANTI-CD27 mAb INCREASES PROLIFERATION IN WT AND CD62L DEFICIENT CD4 ⁺ T CELLS	126
6.5	BB-3103 INHIBITS ANTI-CD27 MEDIATED PROLIFERATION INCREASE IN WT CD4 ⁺ T CELLS	127
6.6	BB-3103 DOES NOT INHIBIT ANTI-CD27 mAb MEDIATED PROLIFERATION INCREASE IN CD62L KO CD4 ⁺ T CELLS	129
DISCUSSION (CHAPTER 6)		130
CHAPTER 7		
7.1	AGONIST ANTI-CD62L mAb INCREASES PROLIFERATION IN WT CD4 ⁺ T CELLS	132
7.2	BB-3103 HAS NO EFFECT ON WT CD4 ⁺ T CELL PROLIFERATION WHEN CD62L IS PROVIDED WITH A LIGAND	134
DISCUSSION (CHAPTER 7)		135

CHAPTER 8

8.1	THE EFFECT OF BB-3103 ON TYROSINE PHOSPHORYLATION OF SIGNALLING MOLECULES	137
8.1.1	p56 ^{Lck} PHOSPHORYLATION IS NOT AFFECTED BY BB-3103	137
8.1.2	ITAM PHOSPHORYLATION ON CD3 ζ IS MODIFIED BY BB-3103.....	140
8.1.3	ZAP-70 PHOSPHORYLATION IS REDUCED BY BB-3103	143
8.2	CALCIUM FLUX IN T CELLS IS MODIFIED BY BB-3103.....	145
8.2.1	BB-3103 AFFECTS HUMAN CD4 ⁺ T CELL PROLIFERATION	145
8.2.2	BB-3103 INCREASES TCR MEDIATED [Ca ²⁺] _i FLUX	147
8.2.3	BB-3103 INCREASES CD62L MEDIATED [Ca ²⁺] _i FLUX	148
	DISCUSSION (CHAPTER 8)	149

CHAPTER 9

9.1	BB-3103 DIFFERENTIALLY AFFECTS HUMAN Th₁ AND Th₂ CD4⁺ T CELL SUBSETS	153
9.1.1	CYTOKINE SECRETION FROM PROLIFERATING ATOPIC AND CONTROL PBMCs TO HOUSE DUST MITE AEROALLERGEN Der P 1	154
9.1.2	NON-ATOPIC PBMC PROLIFERATION TO Der p 1 IN THE PRESENCE OF BB-3103	155
9.1.3	ATOPIC PBMC PROLIFERATION TO Der p 1 IN THE PRESENCE OF BB-3103	157
9.1.4	MODIFIED CYTOKINE SECRETION FROM PBMCs IN THE PRESENCE OF BB-3103	159
9.2	BB-3103 DIFFERENTIALLY AFFECTS HUMAN TH₀ AND TH₂ CLONES.....	163
9.2.1	BB-3103 INDUCES IFN γ PRODUCTION IN Th ₂ CD4 ⁺ CLONES	165
9.3	BB-3103 AFFECTS MURINE POLARISED CD4⁺ T CELLS	168
9.3.1	INDUCTION OF Th ₁ AND Th ₂ TYPE ENVIRONMENT	168
9.3.2	PROLIFERATION OF MURINE Th ₁ AND Th ₂ CD4 ⁺ CELLS.....	170
9.4	EXPRESSION OF CD62L IS MAINTAINED ON Th₁ TYPE CD4⁺ CELLS	172
	DISCUSSION (CHAPTER 9)	173
	CONCLUSIONS	177
	APPENDIX	179
	REFERENCES	185

PUBLICATIONS	203
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LIST OF TABLES

TABLE 1. 1: SUMMARY OF MEMBERS OF THE MMP FAMILY AND THEIR SUBSTRATE SPECIFICITIES. 25

TABLE 1. 2: ENZYMES CAPABLE OF TWO STEP ACTIVATION OF MMPS 29

TABLE 5. 1: SUMMARY OF THE PHENOTYPIC DATA ACQUIRED BY FLOW CYTOMETRY ON PURIFIED CD4⁺ T CELLS 104

TABLE 6. 1: SUMMARY OF THE PHENOTYPIC DATA ACQUIRED BY FLOW CYTOMETRY ON PURIFIED CD62L KO CD4⁺ T CELLS..... 121

LIST OF FIGURES

FIGURE 1. 1: FAMILIES OF ZINC METALLOPROTEINASES.	22
FIGURE 1. 2: DIAGRAMMATIC REPRESENTATION OF MMPS 1, 2, 3 AND 9 SECRETED BY T LYMPHOCYTES.	24
FIGURE 1. 3: DOMAIN ORGANISATION AND PREDICTED FUNCTION OF ADAMS..	31
FIGURE 1. 4: DESIGN OF METALLOPROTEINASE INHIBITORS IS BASED AROUND THE CLEAVAGE SITE OF COLLAGEN MOLECULES.....	34
FIGURE 1. 5: DIAGRAMMATIC REPRESENTATION OF BB-3103.	36
FIGURE 1. 6:STRUCTURE AND SIGNALLING FROM SOME TNFR FAMILY MEMBERS AND THEIR INTRACELLULAR ADAPTER PROTEINS.	41
FIGURE 1. 7: SCHEMATIC REPRESENTATION OF THE L-SELECTIN ADHESION MOLECULE.....	43
FIGURE 1. 8: DIAGRAM REPRESENTING Lck (56KDA).....	52
FIGURE 1. 9: DIAGRAM SHOWING ASSOCIATION OF ONE CD3 ζ TRANSMEMBRANE MOLECULE WITH THE TCR.....	54
FIGURE 1. 10: DIAGRAM OF ZAP-70.....	55
FIGURE 1. 11: OUTLINE OF TCR MEDIATED SIGNALLING PATHWAYS.....	59
FIGURE 3. 1: C57BL/6 SPLEEN PROLIFERATION TO PMA/IONO IN THE PRESENCE OF BB-3103.....	80
FIGURE 3. 2: C57BL/6 SPLEEN PROLIFERATION TO LPS IN THE PRESENCE OF BB- 3103.....	81
FIGURE 3. 3: PROLIFERATION OF B CELLS IN C57BL/6 SPLEEN BY CROSS LINKING BCR IN THE PRESENCE OF BB-3103.	82
FIGURE 3. 4: C57BL/6 SPLEEN PROLIFERATION TO CONA IN THE PRESENCE OF BB- 3103.....	83

FIGURE 3. 5: C57BL/6 SPLEEN PROLIFERATION TO PWM IN THE PRESENCE OF BB-3103.....	84
FIGURE 3. 6: PROLIFERATION OF PURIFIED CD90 ⁺ CELLS TO ANTI-CD3 mAb& ANTI-CD28 mAb IN THE PRESENCE OF BB-3103	86
FIGURE 3. 7: PROLIFERATION OF PURIFIED CD4 ⁺ CELLS TO ANTI-CD3 mAb AND ANTI-CD28 mAb IN THE PRESENCE OF BB-3103.....	87
FIGURE 3. 8: PROLIFERATION OF PURIFIED CD8 ⁺ CELLS TO ANTI-CD3 mAb AND ANTI-CD28 mAb IN THE PRESENCE OF BB-3103.....	88
FIGURE 4. 1: BB-3103 AFFECTS CELL NUMBER IN PROLIFERATION ASSAYS.	93
FIGURE 4. 2: BB-3103 DOES NOT INCREASE ANNEXIN-V-FITC STAINING ON CD4 ⁺ T CELLS	95
FIGURE 4. 3: PROLIFERATION OF EL4 AND JURKAT E6.1 IN THE PRESENCE OF 20 μ M BB-3103.....	97
FIGURE 4. 4: PROLIFERATION OF HT-2 CELLS IN THE PRESENCE OF BB-3103..	97
FIGURE 4. 5: IC ₅₀ CURVE FOR BB-3103 IN CD4 ⁺ T CELLS..	99
FIGURE 5. 1: CYTOKINE SECRETION OF ACTIVATED CD4 ⁺ T CELLS IN THE PRESENCE OF BB-3103:.....	103
FIGURE 5. 2: BB-3103 AFFECTS CD27 EXPRESSION ON CD4 ⁺ T CELLS.....	105
FIGURE 5. 3: BB-3103 AFFECTS CD62L EXPRESSION ON WT CD4 ⁺ T CELLS.....	107
FIGURE 5. 4: BB-3103 DOES NOT AFFECT CD25 EXPRESSION ON WT CD4 ⁺ T CELLS..	109
FIGURE 5. 5: BB-3103 DOES NOT AFFECT CD69 EXPRESSION ON WT CD4 ⁺ T CELLS.	110
FIGURE 5. 6: BB-2116 AFFECTS TNF α AND TNFRII RELEASE FROM HUMAN PBMCs.	111

FIGURE 5. 7: INHIBITION OF PROLIFERATION IS NOT DUE TO INHIBITION OF TNF α RELEASE..	112
FIGURE 6. 1: CD4 ⁺ T CELL PROLIFERATION FROM WT AND CD62L KO ANIMALS IN THE PRESENCE OF BB-3103.....	118
FIGURE 6. 2: CYTOKINE SECRETION OF ACTIVATED CD62L KO CD4 ⁺ T CELLS IN THE PRESENCE OF BB-3103:.....	119
FIGURE 6. 3: CD62L EXPRESSION ON CD62L GENE DEFICIENT CD4 ⁺ T CELLS.....	120
FIGURE 6. 4: BB-3103 DOES NOT AFFECT CD25 EXPRESSION ON CD62L KO CD4 ⁺ T CELLS..	122
FIGURE 6. 5: BB-3103 DOES NOT AFFECT CD69 EXPRESSION ON CD62L DEFICIENT CD4 ⁺ T CELLS.....	123
FIGURE 6. 6: BB-3103 AFFECTS CD27 EXPRESSION ON CD62L DEFICIENT CD4 ⁺ T CELLS.	125
FIGURE 6. 7: SOLUBLE ANTI-CD27 mAb INCREASES PROLIFERATION OF WT CD4 ⁺ T CELLS.	126
FIGURE 6. 8: SOLUBLE ANTI-CD27 mAb INCREASES PROLIFERATION OF WT CD4 ⁺ T CELLS.	127
FIGURE 6. 9: PROLIFERATION OF WT CD4 ⁺ T CELLS IN THE PRESENCE OF SOLUBLE ANTI-CD27 mAb AND BB-3103.....	128
FIGURE 6. 10: PROLIFERATION OF CD62L KO CD4 ⁺ T CELLS IN THE PRESENCE OF SOLUBLE ANTI-CD27 mAb AND BB-3103.	129
FIGURE 7. 1: PROLIFERATION OF WT CD4 ⁺ T CELLS IN THE PRESENCE OF PLATE BOUND ANTI-CD3 mAb AND SOLUBLE ANTI-CD28 mAb WITH SOLUBLE ANTI-CD62L mAbs..	132

FIGURE 7. 2: BB-3103 HAS NO EFFECT ON WT CD4 ⁺ T CELL PROLIFERATION WHEN CD62L IS PROVIDED WITH A LIGAND..	134
FIGURE 8. 1: P56 ^{LCK} PHOSPHORYLATION IS NOT AFFECTED BY BB-3103.	138
FIGURE 8. 2: ITAM PHOSPHORYLATION ON CD3 ζ IS MODIFIED BY BB-3103	141
FIGURE 8. 3: ZAP-70 PHOSPHORYLATION IS MODIFIED BY BB-3103.	144
FIGURE 8. 4: PROLIFERATION OF HUMAN CD3 ⁺ CELLS IS INHIBITED BY BB-3103...	146
FIGURE 8. 5: BB-3103 INCREASES TCR MEDIATED [Ca ²⁺] _i FLUX.....	147
FIGURE 8. 6: BB-3103 INCREASES CD62L MEDIATED [Ca ²⁺] _i FLUX.....	148
FIGURE 8. 7: DIAGRAMMATIC REPRESENTATION OF POSSIBLE INVOLVEMENT OF CD62L IN THE REGULATION OF T CELL ACTIVATION.	152
FIGURE 9. 1: CYTOKINE SECRETION FROM PROLIFERATING ATOPIC AND NON-ATOPIC PBMCs TO Der p 1.	154
FIGURE 9. 2: NON-ATOPIC PBMC PROLIFERATION TO Der p 1 IN THE PRESENCE OF BB-3103.....	156
FIGURE 9. 3: ATOPIC PBMC PROLIFERATION TO Der p 1 IN THE PRESENCE OF BB-3103.....	157
FIGURE 9. 4: MODIFIED CYTOKINE SECRETION FROM PBMCs IN THE PRESENCE OF BB-3103.....	160
FIGURE 9. 5: BB-3103 DIFFERENTIALLY AFFECTS HUMAN Th ₀ AND Th ₂ CLONES....	163
FIGURE 9. 6: CYTOKINE PROFILE FROM AC1.1 AND HA1.7 PROLIFERATIONS IN THE ABSENCE OF BB-3103.	165
FIGURE 9. 7: BB-3103 INDUCES IFN γ PRODUCTION IN HUMAN CD4 ⁺ Th ₂ CLONES..	166
FIGURE 9. 8: INDUCTION OF A Th ₁ OR Th ₂ TYPE ENVIRONMENTS..	169
FIGURE 9. 9: PROLIFERATION OF MURINE Th ₁ AND Th ₂ TYPE CD4 ⁺ T CELLS.....	170
FIGURE 9. 10: CD62L EXPRESSION IS MAINTAINED ON Th ₁ CD4 ⁺ T CELLS.....	172

ABBREVIATIONS

- ³ H-TdR	Tritiated Thymidine
-Ab	Antibody
-ADAM	A Disintegrin and Metalloproteinase
-AICD	Activation Induced Cell Death
-AIDS	Acquired Immunodeficiency Syndrome
-AIM	Activation inducer molecule
-APC	Antigen Presenting Cell
-BCR	B Cell Receptor
-BM	Basement Membrane
-Ca ²⁺	Calcium ion
-CaM	Calmodulin
-ConA	Concanavalin A
-CRAC	Calcium Release Activated Calcium (channels)
-DAG	Diacylglycerol
-DC	Dendritic Cell
-Der p 1	<i>Dermatophagoides pteronyssinus</i> allergen group 1
-EC	Endothelial Cells
-ECM	Extracellular Matrix
-ELISA	Enzyme Linked ImmunoSorbant Assay
-GEF	Guanine Exchange Factor
-GTP	Guanidine Tri-Phosphate
-HRP	HorseRadish Peroxidase
-IAP	Inhibitor of Apoptosis
-IC50	Inhibition concentration at with 50% efficiency
-IFN	Interferon
-IL-	Interleukin
-Iono	Ionomycin
-IP ₃	Inositol triphosphate
-ITAM	Immunoglobulin family Tyrosine-based Activation Motifs
-KO	Knock Out (gene deficient)
-LAT	Linker of Activated T cells
-LHS	Left Hand Side
-LPS	Lipopolysaccharide
-mAb	Monoclonal Antibody
-MAPK	Mitogen Activated Protein Kinase
-Met turn	Methionine turn
-MFI	Mean Fluorescence Intensity
-MHC	Major Histocompatibility Complex
-MIP1	Macrophage Inflammatory Protein 1
-MMP	Matrix Metalloproteinase
-MP	Metalloproteinase
-MT-MMP	Membrane Type Matrix Metalloproteinase
-NIK	NFκB inducing Kinase
-PBMCS	Peripheral Blood Mononuclear Cells
-PBS	Phosphate Buffered Saline
-PGE ₂	Prostaglandin E2
-PIP ₂	Phosphatidyl inositol 4, 5 bi-phosphate
-PMA	Phorbol 12-Myristate 13-Acetate
-PP	Phospho-protein
-PS	Phosphatidyl Serine
-PWM	Pokeweed Mitogen

-PY	Phospho-Tyrosine
-RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
-rbc	Red Blood Cell
-RHS	Right Hand Side
-SCID	Severe Combined Immunodeficiency
-SP	Signal Peptide
-TACE	Tumour Necrosis Factor α Converting Enzyme
-TCR	T Cell Receptor
-Th	T helper
-Th ₀	T helper 0
-Th ₁	T helper 1
-Th ₂	T helper 2
-TLR	Toll-like receptor
-TNF	Tumour Necrosis Factor
-TNFR	Tumour Necrosis Factor Receptor
-TRADD	TNFR Associated Death Domain
-TRAF	TNFR Associated Factor
-VCAM	Vascular Cell Adhesion Molecule
-VIP	Vasoactive Intestinal Peptide
-WT	Wild Type
-Y	Tyrosine
-ZAP-70	Zeta Associated Protein 70kDa
-ZBG	Zinc Binding Group
-Zn ²⁺	Zinc ion

INTRODUCTION

1.1 AIMS OF THESIS

The aims of the research reported in this PhD thesis were to investigate, with the use of a synthetic zinc metalloproteinase inhibitor, the potential role of metalloproteinase enzyme activity in the process lymphocyte activation and any differential functions on Th₁ and Th₂ subsets of CD4⁺ Th cells. Identification of metalloproteinase substrates expressed on lymphocytes and the function of these targets would advance our knowledge of the pathways required for regulating immunological responses.

1.2 INTRODUCTION LAYOUT

The introduction first summarises the knowledge known to date on zinc metalloproteinase members expressed on lymphocytes and then describes structures and function of natural and synthetically manufactured metalloproteinase inhibitors.

The following section describes the role of major substrates for metalloproteinases expressed by lymphocytes, which include TNF superfamily members.

Finally, the introduction ends with a summary of the activation requirements and signalling mechanisms in T lymphocytes.

1.3 ZINC METALLOPROTEINASES

Zinc metalloproteinases are a large family of zinc dependent peptidases (Figure 1.1) (Hooper 1994). All enzymes in this family contain a single zinc ion (Zn^{2+}) in their catalytic site which is indispensable for their enzymatic function, and, chelating Zn^{2+} with chemicals such as EDTA or o-phenanthroline, inactivates all current members of zinc dependent metalloproteinases (Black and White 1998).

As shown in Figure 1.1, each sub-family of zinc metalloproteinases contains many members and the nomenclature and categorisation of various enzymes within the zinc metalloproteinase family is based on analysis of the catalytic site of the enzyme (Hooper 1994). The sequence immediately within the catalytic site responsible for binding the Zn^{2+} defines the first subset to which an enzyme will belong (Zincins: **HEXXH**, Inverzincins: **HXXEH**, Carboxypeptidase: **HXXE** and DD-carboxypeptidase: **HXH** where the residues in bold directly bind Zn^{2+}). Subsequent categorisation is dependent on sites distal from the catalytic site but which are also thought to be capable of binding Zn^{2+} (see Figure 1.1).

In this introduction, matrix metalloproteinases (MMP-matrixin family) and adamalysins (ADAMS- a disintegrin and metalloprotease - closely related to reprotysin family) expressed by lymphocytes will be discussed (shaded in Figure 1.1), since the main aim of this thesis was to investigate the effect of zinc metalloproteinase inhibition in lymphocyte activation. These include MMPs 1, 2, 3 and 9 and ADAMs 10 and 17.

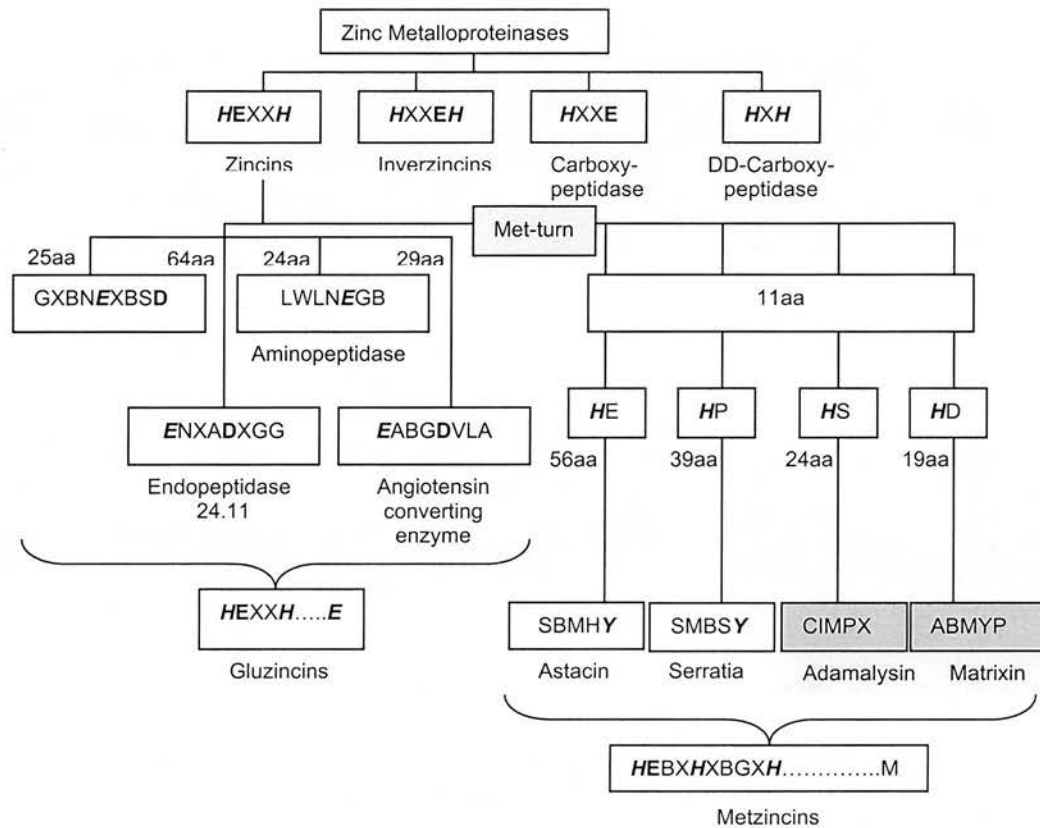


Figure 1. 1: Families of zinc metalloproteinases. The families of the zinc metalloproteinases and their inter-relationships based on the sequence around the zinc binding residues. Italicised bold letters represent positively identified zinc ligands. Bold letters represent residues involved in catalysis. **B** stands for bulky, polar residues. **X** stands for any residue. The number of amino acids(aa) represents the distance between the ligands of the first histidine in the short zinc binding motif (i.e. HEXXH). Met-turn = Methionine turn in protein tertiary structure. Adapted from Hooper 1994.

1.3.1 MATRIXINS / MATRIX METALLOPROTEINASES (MMPs)

As the name suggests, MMPs degrade components of the extracellular matrix (ECM). The controlled enzymatic degradation of ECM is essential in many physiological processes such as development, growth and tissue repair (Streuli 1999). Dysregulated enzymatic degradation of ECM can lead to several pathological conditions such as rheumatoid arthritis, osteoarthritis and autoimmune skin blistering disorders (Kahari and Saarialho-Kere 1997; Shapiro 1998).

There currently are 19 members in the MMP family which are capable of collectively degrading all ECM components (Johnsen *et al.* 1998; Kahari and Saarialho-Kere 1997). These have been classified into subgroups according to their substrate specificity and structure. The subgroups are composed of collagenases, stromelysins, gelatinases, and membrane type MMPs (MT-MMPs) (Table 1.1). Generally, all MMPs contain a signal peptide, a propeptide, a catalytic domain and a haemopexin-like domain linked to the catalytic site by a hinge region also known as the Methionine-turn region (Figure 1.2). The MT-MMPs (or MMP14-17), however, have an additional carboxyl segment which anchors the proteinase in the cellular membrane of the cell (Bode *et al.* 1999). Figure 1.2 shows variations in structure and Table 1.1 summarises the known functions observed between MMP members identified to date.

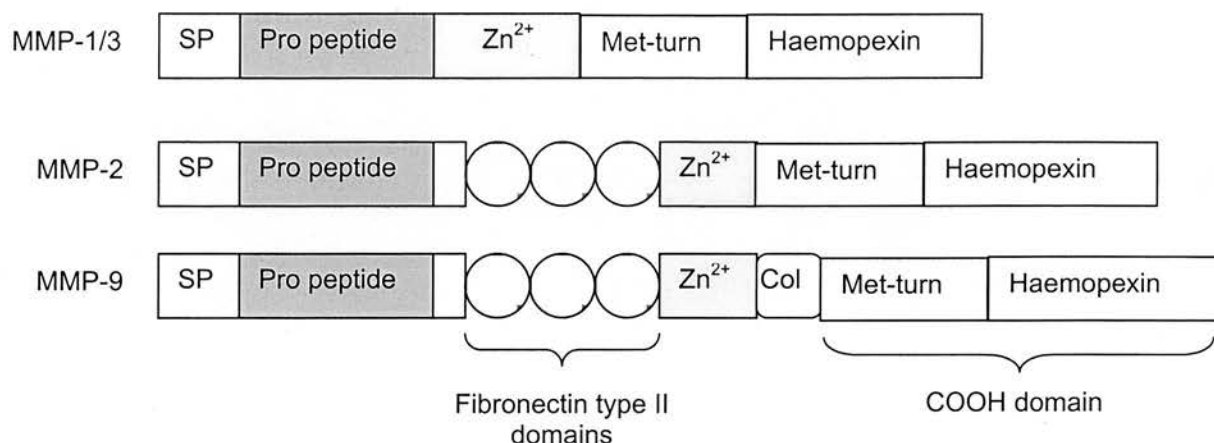


Figure 1. 2: Diagrammatic representation of MMPs 1, 2, 3 and 9 secreted by T lymphocytes. All MMPs contain signal peptides (SP), pro-peptides, catalytic sites (Zn²⁺-binding) and carboxy terminals (COOH). MMPs 2 and 9 contain additional sequences with homology to fibronectin type II (circles). Adapted from Hooper 1994.

Enzyme	MMP number	Matrix substrate or function
Collagenases		
Interstitial collagenase (collagenase-1)	MMP-1	Collagens I, II, III, VII and X, gelatins, entactin, aggrecan, link protein
Neutrophil collagenase (collagenase-2)	MMP-8	Collagens I, II, and III aggrecan, link protein
Collagenase 3	MMP-13	Collagens I, II and III, XI, IV, X, IX, gelatin, laminin, tenascin, aggrecan, fibronectin
Collagenase 4 (<i>X. laevis</i>)	MMP-18	Collagen I
Gelatinases		
Gelatinase A	MMP-2	gelatins, collagens I, IV, V, VII, X and XI, fibronectin, laminin, aggrecan, elastin, large tenascin C, vitronectin, proMMP-9
Gelatinase B	MMP-9	Gelatins collagens IV, V, XIV, aggrecan, elastin, entactin, vitronectin, nidogen
Stromelysins		
Matrylisin	MMP-7	Aggrecan, fibronectin, laminin, gelatins, collagen IV, elastin, entactin, small tenascin-C, vitronectin
Stromelysin 1	MMP-3	Aggrecan, gelatins, fibronectin, laminin, collagens III, IV, IX
Stromelysin 2	MMP-10	Aggrecan, fibronectin, collagen IV, nidogen
Membrane type MMPs		
MT1-MMP	MMP-14	Collagens I, II, III, fibronectin, laminin-1, vitronectin, dermatan sulphate proteoglycan, proMMP-2 and proMMP-13
MT2-MMP	MMP-15	Not known
MT3-MMP	MMP-16	Activates proMMP-2
MT4-MMP	MMP-17	Not known
Others		
Stromelysin 3	MMP-11	Weak activity on fibronectin, laminin, collagen IV, aggrecan, gelatins
Metalloelastase	MMP-12	Elastin
Unnamed	MMP-19	Not known

Table 1. 1: Summary of members of the MMP family and their substrate specificities.

1.3.1.1 MMPs SECRETED BY LYMPHOCYTES

This section describes MMPs which are known to be secreted by lymphocytes, namely MMP-2 and 9, although MMP-1 and 3 have also been identified to be secreted by T lymphocytes. Secretion of these MMPs is upregulated following activation through a variety of factors, namely cytokines and chemokines such as IL-1 (α and β), IL-2, IL-4, RANTES and MIP1 α (Goetzl *et al.* 1996; Leppert *et al.* 1995b), but is more evident upon VCAM-1 dependent adhesion of T lymphocytes to endothelial cells (Romanic and Madri 1994) and integrin dependent adhesion to fibronectin (Xia *et al.* 1996). Other factors which upregulate MMP expression in T lymphocytes are eicosanoids such as prostaglandin E₂ (PGE₂) and vasoactive intestinal peptides (VIPs) (Xia *et al.* 1996; Leppert *et al.* 1995a). The known main functions of T lymphocyte derived MMPs are to hydrolyse matrix components of the basement membrane (BM)-mainly composed of collagens IV, V and fibronectin (Leppert *et al.* 1995b) for subsequent cell migration into inflamed tissue or lymph node (Goetzl *et al.* 1996).

1.3.1.1.1 MMP-1 (COLLAGENASE-1)

MMP-1 is capable of degrading collagens type I, II, III, VII, VIII, X; aggrecan, serpins, gelatins, entactin and α 2-macroglobulin (Kahari and Saarialho-Kere 1997; Shapiro 1998). MMP-1 is expressed by lymphocytes as well as a variety of other cells including fibroblasts, keratinocytes, chondrocytes, monocytes and macrophages and hepatocytes.

1.3.1.1.2 MMP-2 AND MMP-9 (GELATINASES A AND B)

Gelatinase-A (72kDa) or MMP-2 is expressed by a variety of normal and transformed cells including T lymphocytes. Gelatinase-B (92kDa) or MMP-9 is also produced by T lymphocytes as well as keratinocytes, monocytes, alveolar macrophages, neutrophils and a large variety of malignant cells. In addition to their capacity to degrade gelatin, MMP-2 and MMP-9 also have laminin and nidogen as substrates. MMP-2 is unique in that it is capable of proteolytically activating MMP-9 and MMP-13 by cleaving the pro-peptide and thus releasing mature enzyme (Basset *et al.* 1997) (See section on MMP regulation 1.3.1.2).

1.3.1.1.3 MMP-3 (STROMELYSIN-1)

MMP-3, although expressed at low levels by lymphocytes, is capable of degrading aggrecan, gelatin, nidogen, fibronectin, laminin and collagens type III, IV and IX which are the major components of the vascular BM (Airola *et al.* 1997; Goetzl *et al.* 1996).

From the brief description of MMPs secreted by lymphocytes above and the summary of substrates in Table 1.1, it emerges that a wide range of ECM substrates can be cleaved by very few MMPs due to the broad specificity of each enzyme. The enzymes' specificity for the substrates was thought to rely on the presence of the haemopexin domain (Figure 1.2), however, deletion of this domain in collagenases, although eliminating the capacity to cleave triple helical collagen, did not affect the hydrolysis of gelatin, casein or synthetic substrates (Murphy and Knäuper 1997).

1.3.1.2 REGULATION OF MMPs

Since MMPs are a vast family of enzymes which have the potential to cause severe damage to ECM components, the regulation of MMP activity must be tightly regulated *in vivo*. There are three main events regulating MMP activity-

- (1) regulation of protein expression
- (2) secretion as inactive zymogens
- (3) inhibition by Tissue Inhibitors of MetalloProteinases (TIMPs).

1.3.1.2.1 REGULATION OF PROTEIN EXPRESSION

The transcription of MMP genes is very tightly regulated for the majority of MMPs, and is induced in the presence of inflammatory cytokines such as IL-1 (α and β) IL-2, IL-4 and TNF- α , and growth factors such as PDGF, TGF- α , EGF, basic FGF and NGF. TGF- β_1 , however, represses the transcription of all MMPs (Birkedal-Hansen *et al.* 1993). Individual sensitivity of MMPs to these cytokines varies and is also cell specific. In general however, many factors must be integrated to elicit the transcription of MMPs, since regulation has also been induced with cell-matrix interactions and components of the ECM such as lymphocyte VCAM-1 dependent adhesion to HEV (Bonfil *et al.* 1992; Fridman *et al.* 1992; Romanic and Madri 1994; Werb *et al.* 1989). Other stimuli which induce MMP transcription include calcium flux (Unemori and Werb 1988), ultraviolet light (Angel *et al.* 1985) and cell shape (Werb *et al.* 1986). Taken together, these observations indicate that MMP expression is tightly controlled by various factors which synergise to elicit gene transcription.

1.3.1.2.2 ZYMOGEN SECRETION

MMPs are secreted as inactive zymogens with the pro-peptide intact. A cysteine amino acid contained within the pro-peptide binds to the Zn^{2+} within the catalytic site thus inactivating the enzyme. The removal of the pro-peptide and subsequent activation of MMPs occurs in a two step fashion, firstly, the pro-peptide is cleaved in a 'bait' region, upstream of the cysteine, which induces a conformational change. This conformational change allows the removal of the remaining cysteine containing region of the pro-peptide thus revealing the catalytic site (Nagase 1997).

Table 1.2 below summarises the enzymes capable of activating MMPs by removal of pro-peptide.

Zymogen	Initial step	Final step
ProMMP-1	Trypsin, plasmin	MMP-3, MMP-2, MMP-7, MMP-10
ProMMP-2	MMP-1, MMP-14, MMP-16	MMP-3
ProMMP-3	Trypsin, chymotrypsin, plasmin, leukocyte elastase	N/A
ProMMP-9	MMP-1, MMP-2, MMP-3, MMP-7	MMP-1, MMP2, MMP-3, MMP-7

Table 1. 2: Enzymes capable of two step activation of MMPs

1.3.1.2.3 INHIBITION BY TISSUE INHIBITORS OF METALLOPROTEINASES (TIMPs)

Once MMPs are activated by complete removal of the pro-peptide, they are subject to further regulation by TIMPs. The balance between activated MMPs and TIMPs determines net MMP activity. There are three distinct TIMP molecules, TIMPs 1-3 which exhibit 41-52% sequence homology (Douglas *et al.* 1997) and thus the TIMPs do not seem to differentiate much between various MMPs (Murphy and Willenbrock 1995). Knowledge of expression patterns of TIMPs is limited and has not been investigated in lymphocytes, although the

current understanding is that MMP expressing cells always also express TIMPs (Shapiro 1998). The regulation of TIMP expression, on the other hand, is not fully understood.

TIMP-1 is a 28kDa glycoprotein that preferentially forms a 1:1 non-covalent complex with activated MMP-1 and MMP-3. TIMP-1 is also capable of binding both latent and active forms of MMP-9. Interaction with activated MMP-9 results in proteinase inhibition, whereas interaction with pro-MMP-9, inhibits MMP-3 activation of MMP-9 (Bode *et al.* 1999) (Table 1.2).

TIMP-2 is a non-glycosylated 21kDa protein with high affinity for pro-MMP-2 that also binds to the activated form of MMP-2. TIMP-2 has been reported to block the hydrolytic activity of all activated MMPs (Ponton *et al.* 1991). TIMP-3 differs from other TIMPs as it is localised to the ECM and preferentially binds to ECM components rather than latent or activated MMPs.

TIMPs are, therefore, either capable of inhibiting activation of latent gelatinases or inhibition of active MMPs and form a final regulatory step in the control of MMP activation. Recent reports, however, suggest that the function of TIMPs is not limited to inactivation of MMPs but they may also act as growth factors and may have anti-angiogenic activity (Cawston 1998).

1.3.2 ADAMALYSINS

Adamalysins (ADAMs) form a large group of novel cell surface proteins that combine features of both cell surface adhesion molecules and proteinases (Figure 1.1) (Yamamoto *et al.* 1999). Like MMPs, ADAMs are zinc dependent and can be inhibited by chelating Zn²⁺ with EDTA or o-phenanthroline (Black and White 1998). All ADAMs display a common domain organisation and, as a group, possess four functions; proteolysis, adhesion,

signalling and fusion. Figure 1.3 below shows the common structure of ADAMs and the function of each domain.

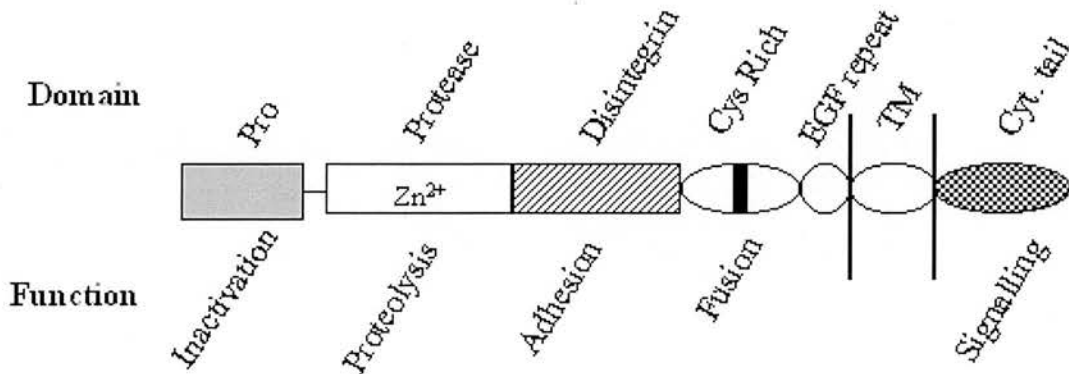


Figure 1. 3: Domain organisation and predicted function of ADAMs. The thick black bar seen in the Cys rich domain depicts a candidate fusion peptide seen only in a limited number of ADAMs. The vertical lines delimiting the TM domain represent the boundaries of the lipid bilayer. Adapted from Black and White, 1998.

To date there are 23 full length ADAM sequences representing cDNAs cloned from mammals, frogs, flies and worms. Among these, 10 are predicted to be active proteinases (ADAMs 1, 8-10, 12, 13, 15, 17, 19 and 20), of which only ADAM10 and ADAM17 have been extensively studied (Black and White 1998).

Since the initial discovery of ADAMs, it has become clear that the proteolytic substrate specificity for the majority of these enzymes is directed against cell surface substrates (Yamamoto *et al.* 1999). This is typified by the two enzymes, ADAM10 and 17 which are expressed by T lymphocytes.

1.3.2.1 ADAMS SECRETED BY T LYMPHOCYTES

1.3.2.1.1 ADAM-10

ADAM 10, first identified in *Drosophila melanogaster* as 'Kuzbanian' and subsequently identified in *Caenorhabditis elegans* as 'SUP-17' was found to be capable of cleaving the Notch receptor from the cell surface and can, thus, modify the early development of the peripheral and central nervous system of these organisms (Sotillos *et al.* 1997; Wen *et al.* 1997).

In mammals, ADAM 10 was identified in bovine brain and can cleave myelin basic protein as well as collagen IV (Howard *et al.* 1996; Millichip *et al.* 1998). There are to date, however, no reports that these two proteins are physiologic substrates for ADAM 10. Notch protein is a substrate for ADAM 10 and since the Notch receptor is highly expressed on T cells (Hoyne *et al.* 2000), ADAM-10 may be required for proper Notch signalling to occur.

1.3.2.1.2 ADAM-17

ADAM 17, highly expressed on T cells, is also known as tumour necrosis factor (TNF) α converting enzyme (TACE) and was first identified as a proteinase which specifically cleaves full length 26kDa TNF α from the cell surface to release the soluble 17kDa form (Black *et al.* 1997; Moss *et al.* 1997). T lymphocytes homozygous for a targeted mutation in the Zn²⁺ binding region of ADAM 17 released 80-90% less soluble TNF α as compared to wild type cells (Black *et al.* 1997).

Various other studies have demonstrated that TNF receptor family members are the primary cell surface molecules identified to be cleaved by ADAM proteinases (Arribas *et al.* 1996;

Hooper *et al.* 1997). These include TNF receptors I and II (Crowe *et al.* 1995) IL-6 receptor (IL-6R) (Mullberg *et al.* 1994), Fas ligand (FasL) (Tanaka *et al.* 1995) and CD30 (Hansen *et al.* 1995). These TNF family members amongst others will be discussed later.

1.3.2.2 REGULATION OF ADAMS

The regulation of ADAMs members is less well understood than MMPs as there is very little evidence for the transcriptional regulation of any of the ADAMs members. The homology between ADAMs pro-domains and MMP pro-domains suggests that enzymes capable of activating MMPs could theoretically also activate ADAMs, although there is no evidence for this yet (Black and White 1998).

TIMP regulation of ADAM activity remains a controversial issue with reports that TIMP-3 is capable of inhibiting TACE (Moss *et al.* 1997) and other reports stating TIMPs are incapable of inhibiting any ADAM family members (Black and White 1998). Inactivating mutations in TACE inhibits TNF α release by 80-90% (Black *et al.* 1997), and the remainder of the release is probably mediated by MMPs although this has yet to be demonstrated.

1.3.3 DESIGN OF METALLOPROTEINASE INHIBITORS

The design of synthetic metalloproteinase inhibitors has focused on two aspects, the cleavage site of the substrate (designing competitive peptides) and the presence of zinc ions in the catalytic site of the enzyme (chelating the Zn²⁺). The initial guide for substrate design of pseudopeptide drugs revolved around screening compound libraries based on the Glycine-Isoleucine and Glycine-Leucine cleavage site in human collagen molecules which are hydrolysed by collagenases (MMP-1, 8, 13). The key to obtaining potent enzyme inhibition was to incorporate potent zinc binding groups (ZBG) within the pseudopeptides (Figure 1.4, below).

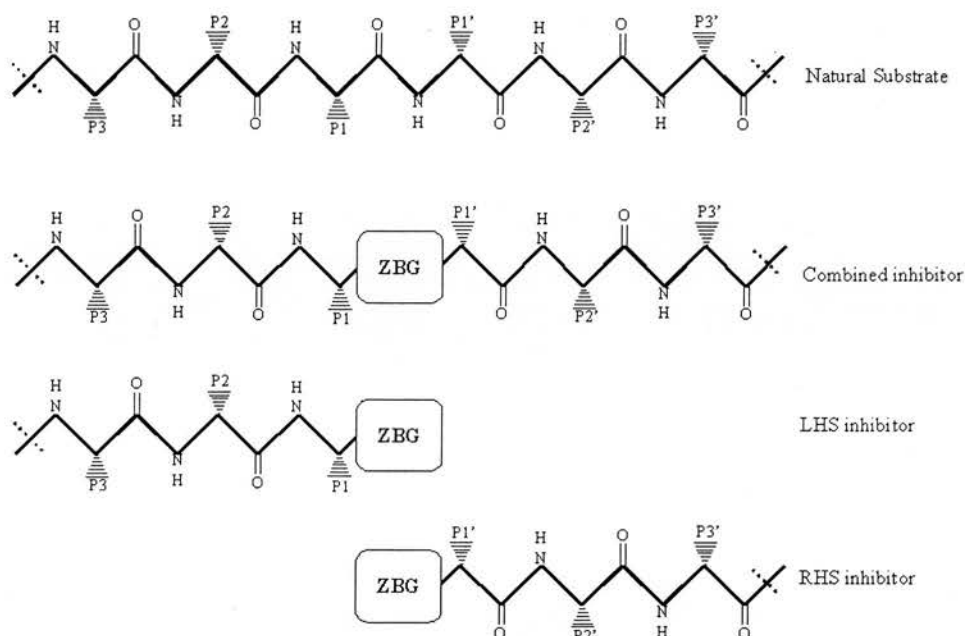


Figure 1. 4: Design of metalloproteinase inhibitors is based around the cleavage site of collagen molecules. Inclusion of a zinc binding group (ZBG) within the pseudopeptide renders the inhibitors much more effective in inactivating the enzyme. LHS= Left Hand Side, RHS= Right Hand Side. Adapted from Moore and Spilburg 1986.

The generation of ZBG containing pseudopeptides also gave rise to three potential drug designs. The inhibitor could mimic both NH₂ and COOH terminals of collagen (combined inhibitor) or either the left (LHS- NH₂) or right hand sides (RHS- COOH) of the collagen molecule (Pratt *et al.* 1998) (Figure 1.4). In 1986, studies showed that ZBG containing RHS inhibitors were more efficient at inhibiting metalloproteinases than either LHS or combined inhibitors (Moore and Spilburg 1986).

The study of potential ZBG which could be used in pseudopeptide molecules has revealed that hydroxamic acid groups were more potent at chelating the Zn²⁺ in the catalytic site of collagenase (MMP-1). The preference of chelating agents is as follows: hydroxamic acid>>

formylhydroxylamine> sulphydryl> phosphinate> aminocarboxylate> carboxylate (Pratt *et al.* 1998).

The P1' group (Figure 1.5) is the major determinant of activity and selectivity of the drugs. For example, small alkyl groups are preferred for the inactivation of neutrophil collagenase, whereas longer alkyl groups or phenylalkyl chains preferentially inhibit stromelysin 1 and gelatinase. The P2' (Figure 1.5) groups can include a wide variety of substituents without affecting inhibitor performance, although aromatic substituents are preferred for activity *in vitro*. The P3' (Figure 1.5) groups can also accommodate a wide variety of substituents, and bulky or aromatic groups have been found to improve inactivation of stromelysin (Pratt *et al.* 1998).

Figure 1.5 below shows the structure of the metalloproteinase inhibitor used in this project, BB-3103, and the concentrations of inhibitor required to inhibit enzyme activity by 50% (IC_{50} values) against various MMPs tested at British Biotech plc.

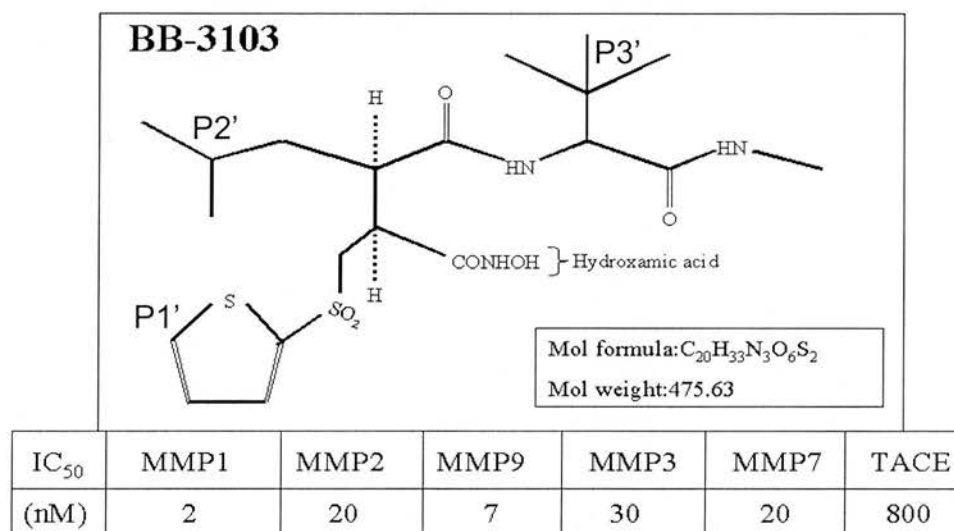


Figure 1. 5: Diagrammatic representation of BB-3103. IC_{50} values tested on various MMPs show the broad range inhibition properties of BB-3103. Adapted from British Biotech BB-3103 technical data information sheet shipped with BB-3103.

1.4 SUBSTRATE CANDIDATES FOR METALLOPROTEINASES EXPRESSED BY LYMPHOCYTES

There are increasing numbers of reports suggesting that membrane bound molecules are shed from the surface of T cells upon activation. These markers include TNF α , TNF receptors 1 and 2, FasL, IL-6 receptor α -chain, CD62-L, CD43 and CD30. Downregulation of these molecules is thought to be orchestrated by zinc-dependent metalloproteinases (Arribas *et al.* 1996; Hooper *et al.* 1997). This introduction gives a brief outline of knowledge gained to date on each of the molecules mentioned above and the importance of molecular shedding from the lymphocyte cell surface upon activation.

1.4.1 TNF α AND TNF RECEPTORS 1 AND 2

TNF α is constitutively expressed on naïve T cells as a 26kDa transmembrane protein and is shed within hours from the surface upon TCR activation as a soluble 17kDa molecule. This process is mediated by TACE or ADAM 17 (Black *et al.* 1997; Moss *et al.* 1997). Shedding of TNF α from the cell surface enhances the trimerisation of soluble TNF α , which consequently becomes biologically active. The TNF α shedding process and subsequent homo-trimerisation suggests that TNF α is capable of not only acting in a paracrine, but also in an autocrine fashion. Soluble TNF α homo-trimers, however, are not as effective as the membrane-bound homo-trimers in triggering a cell receptor-mediated response (Grell *et al.* 1995).

Soluble or membrane bound homo-trimers of TNF α are capable of binding two types of receptors, TNFR1 (p55 or CD120a) and TNFRII (p75 or CD120b), although there are reports showing that TNFRII has lower affinity for soluble TNF α (Grell *et al.* 1995).

Both of these receptors are also capable of being shed, like their ligands, by zinc dependent metalloproteinases (Müllberg *et al.* 1995) or by alternative splicing events (Tanaka *et al.* 1998). Unlike the ligands, however, the soluble forms of receptors are reported to inhibit signalling by competing with membrane bound receptors for TNF α ligands (Wallach *et al.* 1998).

TNFR1 and TNFR2 share homology in their extracellular domains but they differ structurally in the cytoplasmic tail region. TNFR1 contains intracellular 'death domains' (DD), in the cytoplasmic tail which, upon ligation with a homo-trimer ligand, are capable of recruiting and activating two types of adapter proteins (Wallach *et al.* 1998) (Figure 1.6)

The first family of adapter proteins also contain DD and are thus capable of interacting with each other as well as with the cytoplasmic tail of the receptor. TNFR1 binds four such adapter proteins (TRADD, Mort1/FADD, RIP and RAIDD/CRADD) (Figure 1.6) (Wallach *et al.* 1998). Once the adapter proteins interact with TNFR1, they can recruit and activate pro-forms of cysteine proteases known as caspases, namely caspases 2, and 8 (Figure 1.6). The activity of these enzymes are central to all known processes of programmed cell death (Nicholson and Thornberry 1997; Wallach 1997).

The second group of adapter molecules which interact with TNFR1 are those which lack DD. Daxx is such a molecule and is capable of activating a Jun-NH₂ kinase (JNK) which leads to cell death by as yet, unknown mechanisms (Yang *et al.* 1997).

TNFR2, on the other hand, does not contain intracellular DD and, upon ligation with TNF α homo-trimer, associates with TNF receptor associated factors 1 and 2 (TRAF1 and 2) (Figure 1.6). TRAFs are molecules which are capable of recruiting and activating A20, a zinc finger containing adapter protein which can inhibit apoptosis by an unknown mechanism

(Jäättelä *et al.* 1996). TRAFs can also recruit the cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1 and 2). cIAPs inhibits the activation of caspases and thus programmed cell death (Rothe *et al.* 1995). In addition, TNFR associated factors (TRAFs) can activate NF κ B inducing kinases (NIKs), which through a series of phosphorylations, activate NF κ B and subsequent gene transcription leading to cell activation (Beg and Baltimore 1996; Gravelstein and Borst 1998) (Figure 1.6).

There are however, complications to this system since TRADD and RIP are reported to recruit TRAF2 and cIAP1/2 and thus can also inhibit the apoptotic cascade expected by ligation of TNFR1 with TNF α homo-trimers (Shu *et al.* 1996; Wallach *et al.* 1998).

1.4.2 FasL (CD95L/APO1-L)

FasL is a type II membrane protein and member of the TNF family which binds Fas (CD95), a member of TNFR family. FasL is inducibly expressed on T lymphocytes and constitutively expressed on epithelial cells found in the eye and testes thus conferring antigen ignorance (Moulian and Berrih-Aknin 1998).

Upon T lymphocyte activation, FasL is upregulated and subsequently released from the membrane by metalloproteinases to generate a soluble form of FasL (Peter and Krammer 1998). Fas, however, is constitutively expressed on T lymphocytes and is also found in soluble form, but, this isoform is generated through alternate splicing rather than proteolytic activity (Moulian and Berrih-Aknin 1998) and acts as a soluble competitor to membrane bound Fas.

Fas contains cytoplasmic DD and, as in TNFR1 signalling, association of FADD/Mort1 and pro-caspase-8 on the DD of Fas initiates the proteolytic release of active caspase-8 which subsequently leads to the apoptotic caspase cascade (Zhang *et al.* 1998a). Fas/FasL interactions, therefore, initiate cell-mediated cytotoxicity, activation induced cell death (AICD) and confer antigen ignorance (Moulian and Berrih-Aknin 1998).

Mice with null mutations in Fas or FasL (*lpr* and *gld*, respectively) develop systemic autoimmune disease since Fas/FasL dependent deletion of auto-reactive thymic T cells is impaired (Kishimoto and Sprent 1999; Kurasawa *et al.* 1999). These observations indicate that AICD and thymic selection are highly dependent on Fas/FasL interaction (Brunner *et al.* 1995; Ju *et al.* 1995).

In contrast to the death-inducing signalling action of Fas/FasL interaction, some groups have also found that FasL signalling can increase activation and thus proliferation of human T lymphocytes in conjunction with TCR mediated signalling (Alderson *et al.* 1993). These observations may be related to the possibility that Fas, like TNFR1, may recruit TRAF1/2 and thus induce gene transcription and cell survival through NF κ B activation (Figure 1.6).

Like TNF α , proteolytically secreted soluble homo-trimers of FasL, can induce apoptosis in Fas bearing cells but not as effectively as membrane bound FasL. The existence of soluble FasL is, therefore, believed to enable cells to induce apoptosis in para- and autocrine fashion (Kayagaki *et al.* 1995; Tanaka *et al.* 1995).

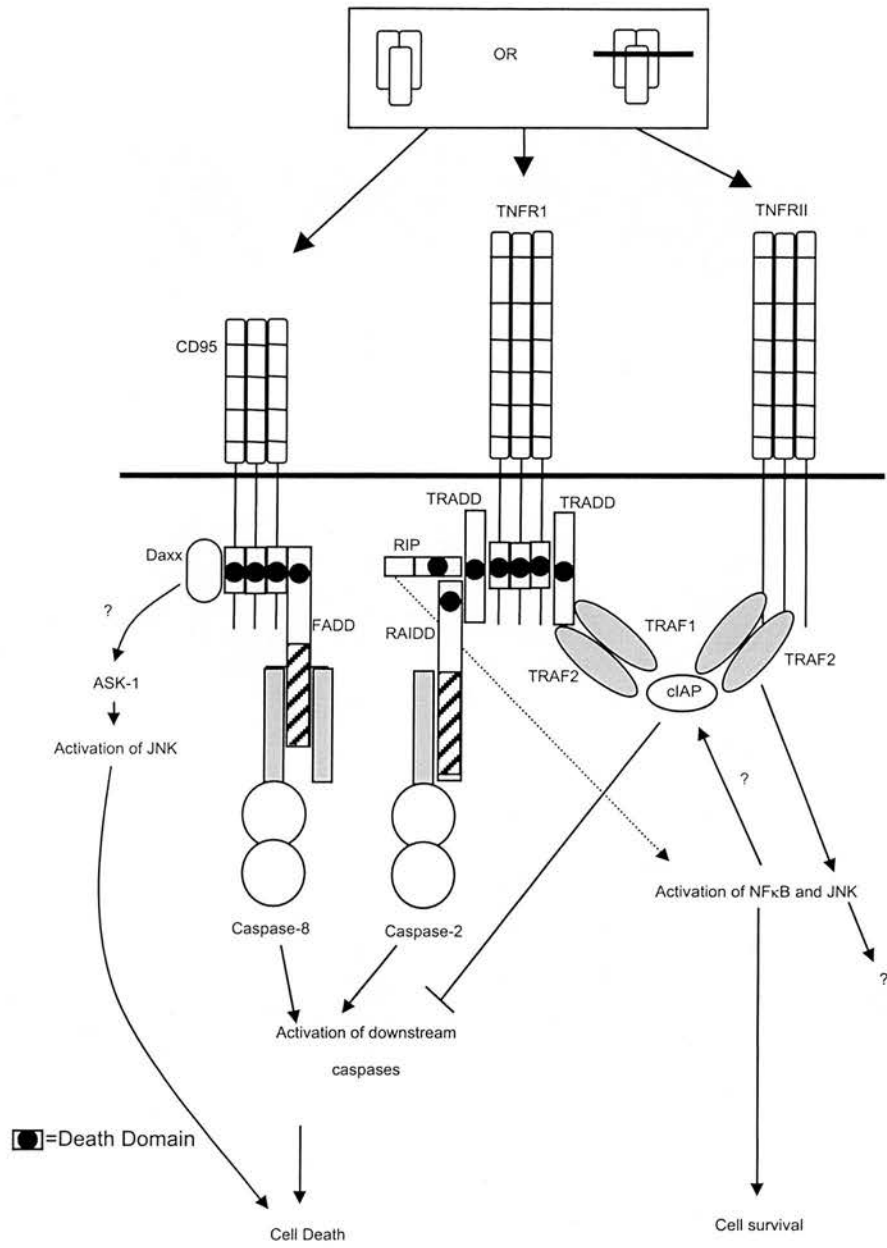


Figure 1. 6:Structure and signalling from some TNFR family members and their intracellular adapter proteins. Adapted from Wallach *at al.* 1998.

1.4.3 IL-6 RECEPTOR COMPLEX (IL-6R)

IL-6R complex is expressed on naïve T lymphocytes and upon IL-6 ligation T lymphocytes undergo enhanced proliferation and differentiation. The IL-6R complex consists of an 80kDa IL-6 binding molecule termed IL-6R α , and a signal transducer, gp130 (Hibi *et al.* 1990; Taga *et al.* 1989). Many cytokine receptors share similar structures to IL-6R α and constitute the type I cytokine receptor super family (Bazan 1990). IL-6R α is also shed from the surface of T lymphocytes by metalloproteinase activity (Müllberg *et al.* 1995).

Contrary to TNFRs and Fas, the soluble form of IL-6R α acts in an agonistic, rather than an antagonistic manner. Indeed, a complex composed of IL-6 and soluble IL-6R α can associate with gp130 and may generate growth signals in cells which express gp130 but not necessarily the IL-6R α -chain. This effect was demonstrated in double transgenic mice which expressed human IL-6 and IL-6R. These mice displayed myocardial hypertrophy (Hirota *et al.* 1995) indicating that the complex of IL-6 and the soluble form of IL-6R α affected heart muscle cells which expressed gp130, whereas IL-6 alone could not induce such an effect.

Signal transducers JAK1, JAK2, and Tyk2 have been shown to associate constitutively with gp130 and to be tyrosine-phosphorylated in response to IL-6, and thus, IL-6 has been shown to activate STAT3 and STAT1 (Fujitani *et al.* 1997; Lai *et al.* 1995; Zhong *et al.* 1994). Phosphorylated, dimerised STATs play pivotal roles in gp130-mediated signal transduction inducing cell growth, differentiation, and survival (Durbin *et al.* 1996; Meraz *et al.* 1996; Kaplan *et al.* 1996a; Thierfelder *et al.* 1996; Kaplan *et al.* 1996b).

In addition to the STAT signal-transduction pathway, the Ras-MAPK pathway may also be activated through binding SHP-2, an adapter protein which recruits downstream signalling

mediators (Grb2 and Sos) (Fukada *et al.* 1996). These will be discussed in the T cell activation section of the introduction.

1.4.4 L-SELECTIN (CD62L)

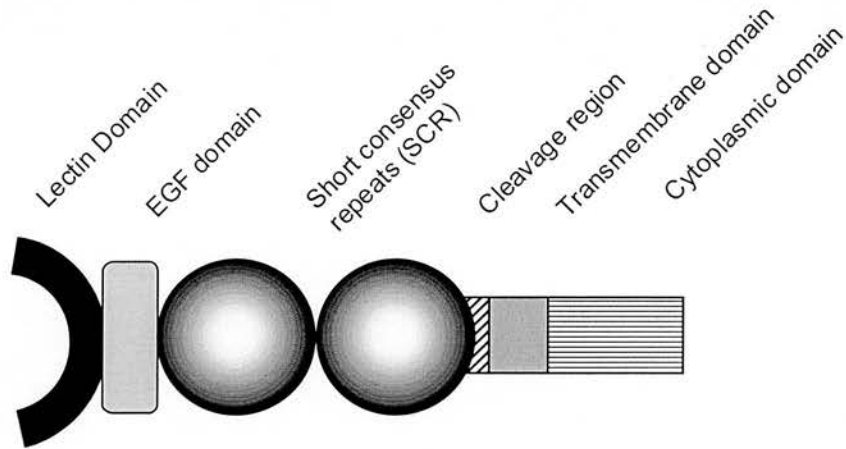


Figure 1. 7: Schematic representation of the CD62L adhesion molecule.

L-selectin (Figure 1.7), also referred to as LAM-1, LECAM-1, Leu-8 MEL-14 (mouse), TQ-1 but more recently CD62L, is a 65kDa transmembrane adhesion molecule highly expressed on lymphocytes, neutrophils and monocytes (Bevilacqua 1993). CD62L is capable of binding heavily glycosylated, fucosylated, sulphated sialated glycoproteins including CD34, Glycam-1 and MAdcam-1 which are expressed on endothelium (see web site: <http://www.ncbi.nlm.nih.gov/PROW/cd/cd62l.htm>). CD62L, therefore, mediates the adhesion events between lymphocytes and high endothelium venules (HEV) as well as leukocyte rolling on activated endothelium at inflammatory sites (Spertini *et al.* 1991; Spertini *et al.* 1992; Tedder *et al.* 1989).

Lymphocytes from CD62L-deficient mice do not bind to peripheral lymph node HEV, thus lymphocyte recirculation cannot occur, resulting in lymph node atrophy (Arbonés *et al.* 1994; Bennett *et al.* 1996). CD62L also plays a role in the attachment of lymphocytes to myelinated tracts of the central nervous system (CNS) and may contribute to the pathogenesis of some demyelinating disorders of the CNS (Huang *et al.* 1991; Kanda *et al.* 1995). In addition to its adhesive function, many studies have investigated CD62L as a signalling molecule specifically in neutrophils. Antibody cross-linking of CD62L, for example, was shown to (1) enhance neutrophil adhesive functions via Mac-1 integrin, (2) potentiate the fMLP (chemo-attractant peptide) and TNF-induced oxidative burst, (3) induce down-regulation of TNF receptors and (4) enhance tyrosine phosphorylation and activation of mitogen activated protein kinase (Richter and Zetterberg 1994; Simon *et al.* 1995; Waddell *et al.* 1994; Waddell *et al.* 1995).

Upon activation of lymphocytes and leukocytes, CD62L is rapidly shed from the surface of the cell (Kishimoto *et al.* 1989) as a soluble molecule 5-12kDa smaller than membrane bound CD62L (Jung and Dailey 1990). The CD62L shedding process can also be initiated in T lymphocytes by activation with phorbol esters such as PMA (Jung and Dailey 1990). Elevated levels of soluble CD62L have been reported in plasma of patients suffering from sepsis, leukaemia and AIDS (Spertini *et al.* 1992) whilst the levels remain abnormally low in patients suffering from acute respiratory distress syndrome (ARDS) (Donnelly *et al.* 1994). The CD62L shedding event upon lymphocyte and neutrophil activation can be inhibited by hydroxamate inhibitors indicating that this process is mediated by a metalloproteinase (Bennett *et al.* 1996).

Extensive studies on CD62L shedding were performed in neutrophils and showed that binding of CD62L to natural ligands (sulphatides and CD34) or monoclonal anti-CD62L antibody results in CD62L shedding, independent of the state of activation of the cell

(Palecanda *et al.* 1992). It also emerged from similar studies that ligand induced shedding of CD62L, could initiate intracellular signalling cascades such as an increase in $[Ca^{2+}]$, MAPK activation and signalling components such as Rac and Ras (Brenner *et al.* 1996; Brenner *et al.* 1997; Laudanna *et al.* 1994; Waddell *et al.* 1995).

CD62L has been found to be associated with the Src tyrosine kinase p56^{lck} in T lymphocytes (Murakawa *et al.* 1992) although the significance of this association was not fully assessed.

In summary, studies performed on neutrophils show that CD62L can be shed under two different conditions: (1) by activation of cells with either chemo-attractants or inflammatory cytokines and (2) by binding of CD62L to ligands. Both of these shedding processes can be inhibited by metalloproteinases. Further studies in neutrophils demonstrate that CD62L is capable of activating several signalling pathways. Since CD62L is associated with signalling components of T lymphocytes, it is possible that CD62L is capable of regulating activation induced signalling pathways in these cells, although this has not been investigated.

1.4.5 CD43/LEUKOSIALIN

CD43 is a heavily glycosylated transmembrane protein expressed on all lymphocytes (Walker and Green 1999) and extends from the surface of lymphocyte by ~450Å (Cyster *et al.* 1991), compared to the TCR which measures ~70Å (Garcia *et al.* 1996). CD43 is, therefore, thought to be implicated in steric hindrance, inhibiting interactions of TCR/CD3 complex with either MHC:peptide or anti-CD3 antibodies (Sperling *et al.* 1995). Co-ligation of TCR and CD43 with monoclonal antibodies results in increased CD28 independent proliferation since CD28 gene deficient T lymphocytes also proliferate upon CD43 cross-linking (Sperling *et al.* 1995). Unfortunately, no natural ligand for CD43 has yet been identified.

CD43 gene deficient animals show increased proliferative responses to anti-CD3 monoclonal antibodies compared to their wild type counterparts (Thurman *et al.* 1998). Transfecting CD43 deficient T lymphocytes with intact cytoplasmic tails of CD43 restores proliferation to wild type levels (Walker and Green 1999). These results indicate that CD43 is capable of not only regulating T lymphocyte activation by steric hindrance but also by inhibitory signals through cytoplasmic interactions with adapter molecules such as Shc/Grb2 and Vav (Pedraza-Alva *et al.* 1998), and subsequent MAPK activity.

CD43 has also been reported to be proteolytically cleaved from the surface of activated lymphocytes (Bazil and Strominger 1993), especially when it is directly cross-linked with monoclonal antibodies (Sperling *et al.* 1995). The process of CD43 proteolysis has been reported to be mediated by metalloproteinases (Bazil and Strominger 1993) and is inhibited by the metalloproteinase inhibitor 1,10-phenanthroline. The shedding, however, can also be inhibited by serine protease inhibitors suggesting CD43 shedding can be performed by metalloproteinases as well as serine proteases (Bazil and Strominger 1993; Weber *et al.* 1997).

1.4.6 CD30 AND CD27

CD30 is a type I glycosylated transmembrane receptor belonging to the TNFR superfamily, which was originally described as an antigen expressed on Reed Sternberg cells in Hodgkin's disease (Schwab *et al.* 1982). Subsequently, CD30 was shown to be induced upon activation of T lymphocytes (Ellis *et al.* 1993). A soluble form of CD30 (sCD30) was identified in serum, levels of which correlated with viral infection and Th₂ type immune responses (Caligaris-Cappio *et al.* 1995). CD30 has also been reported to be cleaved by metalloproteinases (Hansen *et al.* 1995).

Dual stimulation of CD4⁺ T cells by cross-linking CD30 and TCR induces varied responses including increased proliferation, activation, differentiation and apoptosis depending on the cell type, stage of differentiation and presence of other stimuli (Gruss *et al.* 1994).

Expression of CD30 on CD4⁺ T cells was originally thought to be restricted to Th₂ subsets (Del Prete *et al.* 1995), but later observations proved this to be incorrect and it is now accepted that all CD4⁺ Th populations express CD30 (Hamann *et al.* 1996). The confusion over Th₂ and CD30 expression originated from the ability of IL-4 signalling to induce the expression of CD30 on T cells. Increased expression of CD30 is also observed following CD28 signalling and this occurs in an IL-4 independent fashion (Hamann *et al.* 1996). Maintenance of Th₂ cells in the presence of IL-4 would, therefore, be expected to increase expression of CD30 on those clones.

The ligand for CD30 (CD30L) is expressed on a range of cell types including activated T cells, neutrophils, eosinophils and resting B cells (Horie and Watanabe 1998). It is a member of the TNF family and, although it shares homology with TNF α and FasL, it is not cleaved by metalloproteinases.

CD30 ligation in conjunction with TCR stimulation increases levels of IL-2, TNF α and IFN γ and suggests that CD30, like CD28, is a potent co-stimulator of T cells. CD30 has been shown to enhance proliferation of human and murine peripheral T cells stimulated with sub-optimal levels of immobilised monoclonal anti-CD3 antibody (Gilfillan *et al.* 1998; Smith *et al.* 1993). CD30 ligation results in increased mobilisation of NF κ B to the nucleus (McDonald *et al.* 1995). The activation of NF κ B is mediated by signalling through the cytoplasmic tail of CD30 which, like TNFRII, associates with TRAF 1 and 2 (Aizawa *et al.* 1997) (Figure 1.6).

CD27 is a 240 amino acid type I transmembrane member of the TNFR family which is highly expressed on both resting and activated T lymphocytes (Hintzen *et al.* 1995; Lens *et al.* 1998; Watts and DeBenedette 1999). CD27 is believed to increase T cell proliferation upon binding its ligand, CD70 which is, in contrast, is only transiently expressed on activated T lymphocytes. The mechanism by which CD27 augments T cell proliferation is unknown although some reports state that CD27 can modify ZAP-70 phosphorylation directly (Kobata *et al.* 1994). The outcome of activating T lymphocytes through CD27 in conjunction with TCR, however, does not result in increased levels of IL-2, IL-4 or IL-10, suggesting another mechanism of co-stimulation other than increasing phosphorylation of signalling pathways, namely ZAP-70. CD27 cytoplasmic domain, like CD30 associates with TRAF2 and TRAF5 (Akiba H *et al.* 1997) which eventually lead to NF κ B and JNK activation. This may suggest that the mechanisms of co-stimulation that CD30 and CD27 use may be similar. CD27 also associates with a pro-apoptotic molecule (Siva) (Prasad *et al.* 1997) and therefore has the potential regulate cell death. Lowering the threshold of apoptosis may, therefore, be the mechanism by which CD27 acts as a costimulatory molecule rather than increasing cytokine production. CD27 has not been directly identified as a target for metalloproteases but increased levels of soluble CD27 in biological fluids in patients suffering from a variety of immunopathological diseases has been reported suggesting activated T cells may release a soluble form of CD27 (Loenen *et al.* 1992).

1.5 MECHANISMS AND REQUIREMENTS FOR CD4⁺ T CELL ACTIVATION

Activation of T lymphocytes is essential for mounting an efficient immune response, and this process requires at least two stimuli; the primary stimulus is controlled by the T cell antigen receptor (TCR) interacting with an antigenic peptide bound to Major Histocompatibility Complex class I (MHC class I) for CD8⁺ T cells or MHC class II (for CD4⁺ T cells) on the surface of an antigen presenting cell (APC).

The secondary stimulus, or co-stimulation, can be delivered through a variety of receptor ligand pairs of which the interaction of B7.1 (CD80)/B7.2 (CD86) molecules on the APC with CD28 and CTLA-4 receptors on T lymphocytes is the most important. Engagement of B7 molecules with CD28 and/or CTLA-4 appears to be fundamental since in the absence of this ligand interaction, TCR stimulation fails to induce a T cell response and leads to anergy or AICD (Radvanyi *et al.* 1996).

CD28 gene deficient animals, however, are capable of eliciting normal immune response to viral infections (Shahinian *et al.* 1993) and reject allogeneic skin grafts (Sperling *et al.* 1995). Administration of CTLA-4-Ig, which blocks CD80 and CD86 signalling by competitively binding CD80 and CD86, to immunised animals induces a profound reduction in Th₁ cytokine-producing cells. However, antigen-reactive T cells are still capable of expansion in CTLA-4-Ig treated animals, although the degree of expansion is reduced by 50% compared with that in control Ig-treated animals (Judge *et al.* 1996). These data suggest the presence of other co-stimuli which may also be vital in eliciting effective T lymphocyte activation.

These different interactions initiate a series of signalling cascades which are transduced from the cell membrane to the nucleus, initiating gene transcription and subsequently, T lymphocyte activation.

1.5.1 TCR COMPLEX: STRUCTURE AND KINETICS

There are two major types of TCR found in T cells: $\alpha\beta$ and $\gamma\delta$. This thesis describes $\alpha\beta$ TCR and signalling events derived from this TCR type.

The TCR is found as a complex consisting of the polymorphic TCR α and β subunits which contain the antigen binding site, non-covalently associated with the invariant chains of the CD3 complex ζ , γ , δ , ϵ and ζ : ζ homo-dimer chains (Weiss 1993). The CD3 chains represent the signal transducing complex although they do not possess intrinsic enzymatic activity. Instead, CD3 chains display, in their cytoplasmic tails, motifs called ITAMs (immunoglobulin family tyrosine-based activation motifs) (Osman *et al.* 1996; Weiss and Littman 1994).

ITAMs are capable of being tyrosine phosphorylated and, as a consequence are capable of recruiting protein tyrosine kinases (PTK) (see Figure 1.11 at the end of the signalling section) which transduce signals to the T lymphocytes. Although the biochemical signalling processes occurring after TCR:MHC complex interaction are well understood, there remain unresolved questions about the initial triggering event (Lanzavecchia *et al.* 1999).

The initial requirement for T cell activation is generally accepted to involve TCR complex (TCR:CD3) aggregation on the T cell surface (Boniface *et al.* 1998; Grakoui *et al.* 1999). Recent evidence confirmed that aggregation of several TCRs on the T cell surface could promote concentration of lipid and kinase rich 'rafts' or 'microdomains' (Viola *et al.* 1999). The rafts contain key signalling components such as Src (Lck) and Syk (ZAP-70) tyrosine

kinases, as well as adapter proteins (LAT and SLP-76) (Simons and Ikonen 1997; van Leeuwen and Samelson 1999; Zhang *et al.* 1998b). Concentration of the signalling components at the site of TCR engagement with MHC:peptide complex would thus promote and enhance the signalling to the T lymphocyte. Disruption of the rafts leads to decreased phosphorylation of CD3 ζ chain and PLC γ 1, and also attenuated Ca²⁺ mobilisation within the lymphocyte (Montixi *et al.* 1998).

Despite the interaction between a T cell and an APC presenting relevant antigen lasting several hours, the interaction between one TCR and one MHC:peptide complex has a half life of 10 seconds (Davis *et al.* 1998). The long lasting contact between a T cell and an APC is absolutely necessary for the sustained signalling which maintains gene transcription and to promote cell cycle progression (Timmerman *et al.* 1996). Valitutti *et al.*, showed that approximately 100 MHC:peptide complexes were capable of sequentially triggering over 20000 TCRs on one T lymphocyte suggesting that the MHC:peptide complexes are capable of engaging and triggering, but also dissociating from one TCR to become available for another similar interaction (Valitutti *et al.* 1995).

Upon correct TCR/MHC:peptide complex engagement and subsequent dissociation, the triggered TCR is internalised and degraded in an antigen dose dependent fashion (Viola and Lanzavecchia 1996). High affinity mitogenic monoclonal antibodies to CD3 which induce TCR mediated signalling events, are, therefore, much less effective at triggering TCR at low concentrations since they display a stoichiometry of 1:1 and induce a single cycle of activation rather than serial triggering events (Viola and Lanzavecchia 1996). T cell activation using monoclonal anti-CD3 antibodies, therefore, although very efficient at inducing activation and proliferation at high doses, does not mimic physiological activation processes.



1.6 TCR SIGNALLING PATHWAYS

TCR/CD3 complexes bind MHC molecules bearing relevant peptide. This process of recognition induces raft or microdomain aggregation, thus bringing into close proximity molecules necessary for signal transduction pathways. One of the first events to occur after TCR/CD3 and MHC/peptide contact is activation of the membrane bound Src tyrosine kinase p56^{Lck} (Chan and Shaw 1996).

1.6.1 SRC KINASES AND p56^{Lck} ACTIVATION

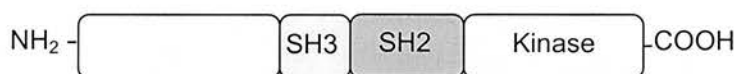


Figure 1. 8: Diagram representing Lck (56kDa). The amino terminal contains a myristilated Lck specific sequence which enables the Src kinase to remain membrane bound. The carboxy terminal sequence contains SH3, SH2 and the enzyme domain of the kinase.

The importance of Lck (Figure 1.8) in T cell activation was highlighted by the finding that mice deficient for Lck suffer from severe combined immune deficiency (SCID) (Molina *et al.* 1992). Another T cell Src tyrosine kinase, Fyn, closely related to but less well characterised than Lck is not critical for T cell activation since Fyn deficient animals still produce normal numbers of antigen responsive peripheral T cells (Stein *et al.* 1992).

Until very recently, Src family tyrosine kinases (including Lck) were thought to be negatively regulated by phosphorylation of tyrosine 527, a critical C-terminal tyrosine residue (Cartwright *et al.* 1987; Kmiecik and Shalloway 1987). Phosphorylation of Y-527 maintains Lck in an inactive state whilst dephosphorylation activates the kinase. Dephosphorylation of Lck is generally accepted to be regulated by the tyrosine phosphatase CD45 since there is increased phosphorylation at the inhibitory site of Src kinases in CD45 deficient mice

(McFarland *et al.* 1993; Sieh *et al.* 1993). Resting T cells, however, also express the tyrosine phosphatase CD45, and Lck is predominantly found dephosphorylated, and, therefore, in an active state (Ostergaard and Trowbridge 1990).

Very recent evidence suggests that Lck activity is also regulated by the transmembrane costimulator molecule, CD28. The SH3 domain of Lck (Figure 1.8) was shown to bind the proline rich region of CD28 cytoplasmic tail. Deletion of either Lck SH3 or mutation of the proline rich region within the cytoplasmic CD28 tail abrogated Lck activity. The interaction of Lck with the proline rich region of the CD28 cytoplasmic tail was found to enhance Lck activation and thus, these data support an additional model of Lck activation (Holdorf *et al.* 1999).

Regulation of Lck activation solely by CD45 seems an unlikely situation since resting T cells contain large amounts of dephosphorylated Lck isoforms, and yet the cells remain quiescent. Involvement of CD28 is appealing since aggregation of the rafts or microdomains to the contact surface of the T cell and APC, would bring both CD28 and activated Lck into close proximity enabling interaction and activation of Lck.

1.6.2 IMPORTANCE OF CD3 ζ ITAM PHOSPHORYLATION

CD3 ζ homo-dimers contain 6 ITAM sequences and, soon after ligation of TCR with MHC:peptide complex, these become phosphorylated by activated Lck (Cantrell 1996) (Figure 1.9 and Figure 1.11).

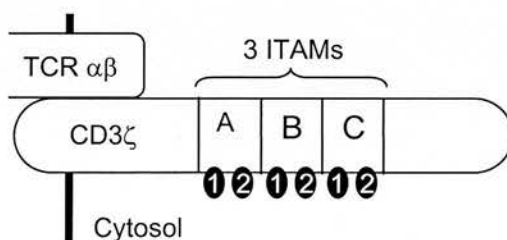


Figure 1. 9: Diagram showing association of one CD3 ζ transmembrane molecule with the TCR. Each CD3 ζ molecule contains 3 ITAM sequences, each with 2 tyrosines (represented by ●). One CD3 ζ molecule thus contains 6 possible phosphorylation sites.

There are many reports of varied ITAM phosphorylation patterns which depend on the activation potential of the MHC:peptide complex (Kersh *et al.* 1998; Madrenas *et al.* 1995; Sloan-Lancaster *et al.* 1994). Most of these studies used altered peptide ligands (APL) and have reported that a strong agonist MHC:peptide complex, capable of eliciting elevated T cell proliferation, induces elevated amounts of CD3 ζ tyrosine phosphorylation as compared to weak agonist MHC:peptide complex which induces 5 fold less proliferation (Kersh *et al.* 1998).

The difference between strong and weak agonist peptide:MHC complexes lies in the pattern of CD3 ζ ITAM phosphorylation. Strong agonist peptides will promote phosphorylation of all six tyrosine residues on a single CD3 ζ molecule (A1 \rightarrow C2) whereas, weaker agonists promote phosphorylation of only the first three tyrosine residues (A1 \rightarrow B1). These patterns of phosphorylation give rise to phospho-isoforms with different molecular weights; p23 and p

21 respectively which can be visualised by electrophoresis and western blotting (Kersh *et al.* 1998).

1.6.3 RECRUITMENT AND ACTIVATION OF ZAP-70



Figure 1. 10: Diagram of ZAP-70. ZAP-70 contains two amino SH2 domains and a carboxy terminal enzyme. One SH2 domain binds to one phospho-tyrosine on one ITAM from CD3 ζ

ZAP-70 (zeta/ ζ associated protein of 70kDa (Figure 1.10)) is a member of the Syk tyrosine kinase family which acts downstream of phosphorylation of ITAMs on CD3 ζ . Both tyrosine residues within each ITAM (A, B or C) on CD3 ζ must be phosphorylated for ZAP-70 to efficiently dock onto a CD3 ζ subunit (Figure 1.9). The efficient interaction between ZAP-70 and phospho-ITAM sequences subsequently influences the downstream TCR-mediated effector functions (Chan *et al.* 1994a). ZAP-70 interacts with the phosphotyrosine residues of the ITAMs through the two SH2 domains (Figure 1.10) and consequently, ZAP-70 becomes activated by phosphorylation of two tyrosine residues (Y-315 and Y-319). ZAP-70 activation process is mediated by activated Src tyrosine kinase Lck (Iwashima *et al.* 1994; Weil *et al.* 1995)(Figure 1.11).

Mice lacking ZAP-70, similarly to the Lck deficient animals, demonstrate a SCID phenotype, which illustrates the critical role ZAP-70 has in TCR mediated signalling (Chan *et al.* 1994b; Roifman 1995).

Upon activation, ZAP-70 phosphorylates LAT and SLP-76, adapter molecules which transduce signals further.

1.6.4 ADAPTER MOLECULES

1.6.4.1 LAT (LINKER FOR ACTIVATION OF T CELLS)

LAT (previously known as pp36/38) is a transmembrane adapter glycoprotein expressed in T cells which is readily phosphorylated by ZAP-70 and is found associated with CD4 in CD4⁺ T cells (Cantrell 1996) (Figure 1.11). Phosphorylated LAT recruits SH2 bearing signalling molecules to the membrane, such as PLC γ 1 and Grb2 (van Leeuwen and Samelson 1999). From studies in Jurkat J.CAM2 cells deficient in LAT, it has become clear that the primary function of LAT is to mediate the coupling of TCR and PLC γ 1-Ca²⁺ and Ras signalling pathways (described later in this introduction) (Finco *et al.* 1998). Association of Grb2 to phosphorylated LAT however provides a link to MAPK pathway through Sos/Ras activation.

1.6.4.2 SLP-76

SLP-76, is also an adapter protein expressed in T cells which binds LAT/Grb2 complex and is then phosphorylated by activated ZAP-70 (Figure 1.11). SLP-76 contains three NH₂ tyrosine phosphorylation sites which upon TCR signalling recruits guanine exchange factor (GEF) Vav which induces an activation of NFATc and thus an increase in IL-2 gene transcription (Bubeck Wardenburg *et al.* 1998). Studies in Jurkat cells deficient in SLP-76 demonstrated defects in activation of PLC γ 1, IP₃ accumulation, and Ras activation.

The adapter proteins are therefore responsible for linking TCR mediated events to appropriate signalling cascades.

1.6.5 PHOSPHOLIPASE C γ 1 (PLC γ 1) AND DOWNSTREAM EVENTS

After successful ZAP-70 docking and phosphorylation, the Syk kinase tyrosine phosphorylates and activates LAT bound PLC γ (Cantrell 1996).

Activated PLC γ hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂) and thereby generates inositol polyphosphates (IP₃) and diacylglycerols (DAG), which allow TCR mediated signalling to regulate both intracellular calcium levels and the serine/threonine kinase family of protein kinase C isozymes (PKC) respectively ((Cantrell 1996) and Figure 1.11).

1.6.6 CALCIUM

The presence of elevated IP₃ concentrations within the T lymphocyte evokes a rise in [Ca²⁺]_i within the cells. The first phase of the [Ca²⁺]_i rise is generally thought to be mediated by IP₃ binding to receptors in the endoplasmic reticulum (ER) membrane and opening Ca²⁺ channels that releases Ca²⁺ into the cytosol (Imboden and Stobo 1985). The second phase is considerably less well understood and is initiated by the depletion of Ca²⁺ within the ER stores. The second phase consists of prolonged influx of Ca²⁺ across the plasma membrane through Calcium Release Activated Calcium channels (CRAC channels) leading to the activation of the serine/threonine phosphatase calcineurin, via a calmodulin:Ca²⁺ intermediate. Dephosphorylation of inactive transcription factors by calcineurin promotes their translocation across the nuclear membrane where they combine with nuclear elements to help drive the expression specific genes (Figure 1.11). Some reports state that Ca²⁺ influx must be sustained for over 30 minutes to commit T cells to become activated and express the IL-2 gene through NFATc activation (Crabtree and Clipstone 1994; Goldsmith and Weiss 1988; Negulescu *et al.* 1994 and Figure 1.11).

1.6.7 RAS ACTIVATION

Following TCR ligation, Ras is activated through GTP binding either through interaction with Sos or direct effects from DAG. Ras activation leads to phosphorylation and activation of the serine/threonine kinase Raf-1 (Hallberg *et al.* 1994). Raf-1 is capable of phosphorylating mitogen activated protein kinase kinase (MAPKK or MEK). ERK is the principal substrate for MEK and upon activation phosphorylates transcription factors such as Fos and Jun which then translocate to the nucleus. NF κ B activation by ERK is thought to be mediated by phosphorylation of the inhibitor of NF κ B (I κ B) which causes the dissociation of the two molecules, releasing NF κ B now free to translocate to the nucleus (Li and Sedivy 1993).

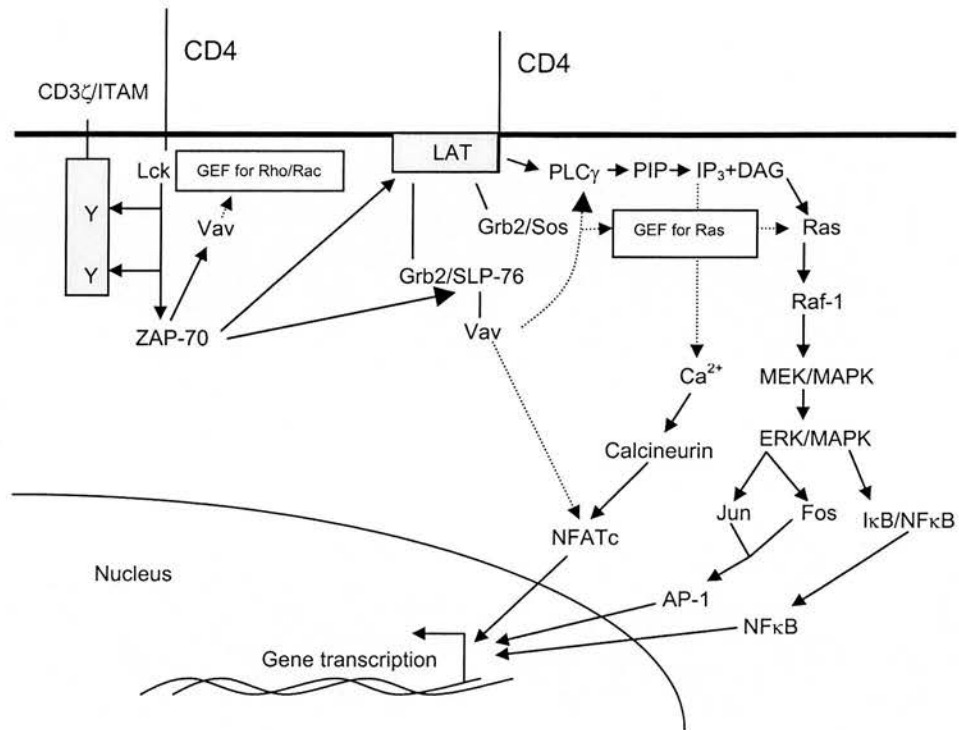


Figure 1. 11: Outline of TCR mediated signalling pathways.

1.7 CD4⁺ T LYMPHOCYTE SUBSETS- TH₁ VS TH₂

In 1986 Mosmann and colleagues identified two types of lymphokine patterns produced by long-term cultured CD4⁺ T-helper (Th) clones that provide help for different arms of the immune system and which have profound counter-regulatory effects on each other (Mosmann *et al.* 1986). These two CD4⁺ T-helper types were named Th₁ and Th₂.

Th₁ cells mainly secrete IL-2, IL-3, IFN γ , TNF α and TNF β thus inducing an inflammatory immune response. Th₂ cells, however, secrete IL-4, IL-5, IL-6 and IL-13, which favour a humoral response associated with allergies. Since then, much work has been focused on defining the different factors which may determine either Th₁ or Th₂ functional differentiation.

1.7.1 ANTIGEN DOSE

Firstly, antigen dose plays a pivotal role in determining the differentiation of precursor CD4⁺ Th cells into either type 1 or type 2 helper CD4⁺ T cells. The first reports of this observation demonstrated that *in vivo*, low and high doses of antigen would promote humoral and cell mediated responses respectively (Liew 1989). This field of research, however, has many conflicting reports (Rogers and Croft 1999). One view which is generally accepted, is that low doses of antigen lead to low numbers of MHC molecules bearing relevant peptides on APCs, resulting in low numbers of ligated TCRs and, consequently, limited phosphorylation of intracellular kinases and reduced mobilisation of calcium in the T cells.

Some reports indicate that *in vitro*, a low degree of T cell activation induces IL-4 production without IFN γ production in resting human memory and effector Th cells (Secrist *et al.* 1995; Carballido *et al.* 1997) and leads to the development of Th₂-cell responses in naïve Th cells

from TCR-transgenic mice (Boutin *et al.* 1997). As many allergens enter the body at low concentrations, this low-dose effect may contribute to the preferential development of Th₂-cell responses as found in allergy. Interestingly, on the basis of the reactivity of allergen-specific Th-cell clones *in vitro*, the study of Carbadillio *et al.* (Carballido *et al.* 1997) suggests that Th cells from patients with atopic allergy require higher doses of allergen to produce IFN γ than their counterparts from non-allergic subjects.

Other, more recent studies, however suggest that a low overall level of stimulation induces short term effector T cells secreting IL-2 with little commitment to either Th₁ or Th₂ differentiation, whereas, a moderate level of stimulation is necessary for T cell survival over an extended time. T cell cultures containing enough peptide to generate long term survival showed that at early time points, lower levels of stimulation could generate Th₁ type differentiation whereas at the same time points, high levels of peptide could favour the Th₂ type differentiation. Over an extended period of time, however, the phenomenon was reversed with low doses and high doses generating Th₂ and Th₁ type T cells respectively (Rogers and Croft 1999).

1.7.2 COSTIMULATION

Secondly, costimulation provided by the APC to the CD4⁺ Th cell has also been found to influence the outcome of Th₁ versus Th₂ differentiation. CD28 deficient mice have impaired immunoglobulin production, but preserved cytotoxic T lymphocyte and delayed type hypersensitivity responses (Shahinian *et al.* 1993). Mice transgenic for soluble CTLA-4-Ig molecule (Lane *et al.* 1994; Ronchese *et al.* 1994) have severe impairment in T-dependent B-cell responses and germinal centre formation, whereas their T cells produce higher levels of IFN γ but lower levels of IL-4.

In three *in vivo* models of Th₂ development (anti-IgD antibody administration, infection with *Heligmosomoides polygyrus*, and infection with *Leishmania major*) CTLA-4-Ig treatment had a profound effect in blocking the generation of IL-4 producing cells (Corry *et al.* 1994; Lu *et al.* 1994; Lu *et al.* 1995). CTLA-4-Ig administration to resistant C57BL/6 mice did not impair their ability to resist infection with *L. major* (Corry *et al.* 1994), and CD28 deficient mice on a resistant background have normal development of Th₁ responses and healing (Reiner and Seder 1995). These results suggest that the initial interaction of CD28 (or possibly CTLA-4) with its B7 ligand is probably required for priming Th₂ responses but is non-essential for some Th₁ responses. The findings that CD86 becomes rapidly upregulated on dendritic cells (Inaba *et al.* 1994) and that both B7 ligands can be induced quickly on a wide variety of APCs bearing MHC class II (Hathcock *et al.* 1994) support their potential role at initiation of any immune response.

Studies using selective blockade of CD80 or CD86 suggest unique roles for the B7 ligands in preferential Th development (Kuchroo *et al.* 1995). Priming or restimulation of TCR transgenic T cells leads to increased IL-4 production in the presence of anti-CD80 (blocking CD80 mediated signalling), but increased IFN γ production in the presence of anti-CD86 (blocking CD86 mediated signalling). When anti-CD80 was used in an experimental autoimmune encephalomyelitis model, disease severity was reduced (and IL-4 production was augmented), whereas the use of anti-CD86 increased disease severity (and augmented IFN γ production) (Kuchroo *et al.* 1995). A role for CD80 in Th₁ development is supported by the finding that CD80 costimulation increases the number of IL-2 mRNA positive cells among the Th₁ and Th₀ clones but is less effective in increasing the number of Th₂ cells that produce IL-4 (Bucy *et al.* 1994). More recent studies, however, using CD80/CD86 gene deficient animals suggest that these molecules contribute to the magnitude of T cell activation, but do not appear to selectively regulate Th₁ versus Th₂ differentiation (Schweitzer *et al.* 1997).

1.7.3 CYTOKINE ENVIRONMENT

Thirdly, the cytokine environment in which naïve T cells encounter antigen influences the differentiation of precursor CD4⁺ Th cells to adopt either a Th₁ or a Th₂ fate. The two main cytokines which play a direct role on the CD4⁺ T cell are IL-4 and IL-12.

IL-12 is a heterodimeric cytokine produced primarily by phagocytic cells such as macrophages and DCs but absent in B cells (Guéry *et al.* 1997) although there are some reports of non germinal centre B cells capable of IL-12 secretion (Schultze *et al.* 1999). Its role in directing Th₁ development in several *in vitro* and *in vivo* systems is now well established (Trinchieri and Scott 1994). Recent advances in IL-12 biology have elucidated some of the mechanisms by which it acts in the initiation and maintenance of immune responses *in vivo*. Some studies (Trinchieri and Scott 1994) highlight the potency of IL-12 in selectively driving the differentiation of Th₁ development but the mechanism of action is less clear: other studies, however, suggest that IL-12 can act directly on T cells to suppress IL-4, whilst some imply that the effects of IL-12 are mediated through the induction of IFN γ . The ability of IL-12 to induce Th₁ responses *in vivo* was initially established in studies of mice infected with *L. major*. Treatment of susceptible BALB/c mice with recombinant (r)IL-12 can inhibit the usual Th₂ response and promote the successful outgrowth of Th₁ effector cells (Heinzel *et al.* 1993; Sypek *et al.* 1993). The importance of endogenous IL-12, however, is highlighted in murine models of candidiasis and toxoplasmosis, in which neutralisation of IL-12 abrogated Th₁ development and impaired survival while promoting Th₂-type responses (Gazzinelli *et al.* 1994; Romani *et al.* 1994). Most studies support the notion that the primary effect of IL-12 in inhibiting initiation of a Th₂ response is indirect, through its induction of IFN γ , but that IL-12 is capable of suppressing IL-4 production directly in some CD4⁺ subpopulations.

Sources of IL-4 which prime Th₂ differentiation has been extensively reviewed (Coffman and von der Weid 1997). In the murine *L. major* model, there is a burst in IL-4 transcription from CD4⁺ cells four days after infection in both susceptible and resistant mice (Reiner *et al.* 1994). Similar findings were made in the schistosome egg granuloma and intestinal helminth models (Svetic *et al.* 1993; Wynn *et al.* 1993). In adoptive transfer studies with IL-4 gene deficient mice, CD4⁺ rather than CD4⁻ cells were the primary source of IL-4 for Th₂ mediated responses (Schmitz *et al.* 1994). A potential source for early IL-4 production by a CD4⁺ cell population includes NK1.1⁺ CD4⁺ cell which was the predominant source of IL-4 in one model of *in vivo* T-cell activation (Yoshimoto and Paul 1994). A pre-activated T cell as primary source of IL-4 is also supported by studies in which CD62L^{low} cells were able to produce the IL-4 that drove the CD62L^{high} cells to become Th₂-like (Gollob and Coffman 1994). Since recent evidence suggests low level of CD62L expression primarily distinguish Th₂ from Th₁ (CD62L^{high}) cells (Wely *et al.* 1999), it is not surprising that CD62L^{low} cells are capable of inducing IL-4 secretion of naïve (CD62L^{high}) CD4⁺ T cells.

Established Th₁ and Th₂ lines from TCR transgenic mice are long-lived and retain their cytokine phenotype after being adoptively transferred into normal recipients (Swain 1994). These cells may be an important source of primary cytokines during a chronic immune response that educates newly activated naïve, precursor Th cells during epitope spreading.

MATERIALS AND METHODS

All buffers and reagent sourcing is indicated at the rear of this chapter in Section 2.5. All reagents were purchased from Sigma unless otherwise stated in brackets after the reagent. Buffers underlined are described in detail in Section 2.5.

2.1 CELL ISOLATIONS AND PURIFICATIONS

2.1.1 MURINE SPLEEN

Isolation of C57BL/6 spleens were used either for proliferation assays or further cell isolation. Spleens were excised from 6-8 week old females, dissected into 2mm sized cubes and placed in 5ml PBS. Tissues were ground between 2 frosted glass slides until a homogenous cell suspension was obtained. Cell suspensions were passed through 70 μ m sterile gauzes and were centrifuged at 100 x g for 7 minutes at room temperature. Cell pellets were resuspended in 1ml ice cold Tris-NH₄Cl red blood cell (rbc) lysis buffer (1ml rbc lysis buffer per spleen) and left on ice for 5 minutes. Cell suspensions were washed three times in PBS by centrifugation at 300 x g and counted using a haemocytometer chamber with 0.4% trypan blue (Sigma) exclusion. Cells were resuspended at 10⁶/ml in complete DMEM (Sigma) until further use. Using this method typically isolated between 100-150x10⁶ cells per spleen depending on the age of the animal. Cells could be purified further using MACS isolation methods described below.

2.1.2 HUMAN PBMC ISOLATION

Human peripheral blood mononuclear cells (PBMCs) were isolated by centrifugal separation over Histopaque-1077 (Sigma). Briefly, blood was obtained from adult volunteers by intravenous puncture into heparinised syringes (5000 units Heparin sodium salt (Sigma) /ml of blood) . The blood was diluted in a 1:1 ratio with room temperature PBS, layered onto 15mls room temperature Histopaque-1077 (Sigma) in 50 ml conical centrifuge tubes

(Falcon) and centrifuged at 800 x g for 20 minutes at room temperature. The mononuclear cell layer was gently aspirated and washed 3 times with 3 volumes PBS by centrifugation at 600 x g for 15 minutes at room temperature. Cells were resuspended in complete RPMI-1640 (Sigma) and counted using a haemocytometer chamber with 0.4% trypan blue (Sigma) exclusion. PBMC numbers typically recovered using this method ranged from 1-2.5x10⁶ cells/ml of blood.

Cells could be purified further using MACS isolation methods described below.

2.1.3 CELL PURIFICATIONS

CD3⁺, CD4⁺, CD8⁺, or CD90⁺ expressing cells were separated from whole spleen suspensions or PBMCs (except CD90⁺) by positive selection to generate enriched cell populations. This was performed using a magnetic cell separation method (MACS) (Miltyeni Biotech, Surrey, U.K.) according to the manufacturer's instructions. Briefly, single cell suspensions were generated as described above (for mouse spleen or human peripheral blood) with the final resuspension at 10⁷ cells per 90µl in MACS buffer (see section 2.5) prior to a 6°C, 15 minute incubation with 10µl antibody coated microbeads (Miltyeni Biotech) per 10⁷ cells. Excess microbeads were removed by washing once in 50 mls MACS buffer and the cell pellet resuspended in 500µl MACS buffer. Labelled cells were allowed to pass through a pre-rinsed (6mls MACS buffer) magnetised positive selection column (Miltyeni Biotech) by gravity flow. The selection column was washed with 3 bed volumes of MACS buffer (9mls total), removing unlabelled cells. The column was demagnetised and cells bearing the antigen of interest (i.e. CD3, CD4, CD8 or CD90) were expelled from the column in 5mls MACS buffer by inserting the column plunger. Mouse cells were washed in 50 mls complete DMEM (Sigma), human cells were washed in 50mls complete RPMI-1640 (Sigma) and counted using a haemocytometer with 0.4% trypan blue (Sigma) exclusion. Using this method of purification typically generated ≥90% pure populations as assessed by flow

cytometric analysis for T cell markers such as TCR or antigen used for separation (CD3, CD4, CD8 or CD90).

2.2 IMMUNOLOGICAL ASSAYS

2.2.1 MITOGEN PROLIFERATION

Polyclonal mitogens used in this study were phorbol 12-myristate 13-acetate (PMA) (Sigma) with ionomycin (Sigma), Concanavalin A (Con A) (Sigma), Pokeweed mitogen (PWM) (Sigma), *E. coli* lipopolysaccharide (LPS) (serotype 055:B5) (Sigma) and anti- μ -chain IgM F(ab')₂ (Jackson ImmunoResearch, USA).

Briefly, single cell suspensions were obtained as previously described for mouse spleen and plated out at 2.5×10^5 cells/well in tissue culture treated 96 round bottom wells (Costar) in a final volume of 200 μ l. Mitogens were added to the wells at pre-determined optimal concentrations, diluted in complete DMEM (Sigma) (PMA and Ionomycin- 0.1 μ g and 0.5 μ g/ml respectively, Con A - 2.5 μ g/ml, PWM - 1 μ g/ml, LPS - 5 μ g/ml, anti- μ -chain IgM F(ab')₂ - 20 μ g/ml). BB-3103 inhibitor was added at a range of concentrations (0.02 μ M, 0.2 μ M, 2 μ M and 20 μ M) to appropriate wells before addition of mitogens. Samples were incubated at 37°C in a humidified incubator with 5% CO₂ for 72 hours. Proliferation was assessed by addition of excess (1 μ Ci/well) tritiated thymidine diluted in PBS (³H-TdR- Amersham Pharmacia Biotech, Amersham, U.K.) for the last 6 or 16 hours of culture or overnight (as stated in the text). Plates were harvested onto glass fibre filtermats (Wallac, Milton Keynes, U.K.) with an automated 96 well plate harvester (Tomtec, Harborview, USA), dried and heat-sealed into plastic bags (Wallac, Milton Keynes, U.K.) with 10ml of scintillation fluid (Wallac, Milton Keynes, U.K.). ³H-TdR incorporation was assessed using a liquid scintillation counter Betaplate 1205 (Wallac, Milton Keynes, U.K.) and associated software. Counts per minute (cpm) observed per sample were indicative of the extent of

proliferation. All samples were tested in triplicate or sextuplet and each experiment repeated three times unless otherwise stated in the Figure legends.

2.2.2 POLYCLONAL TCR STIMULATION USING PLATE BOUND AND SOLUBLE ANTIBODIES

Stimulation of purified T cell populations was performed using immobilised monoclonal antibodies against CD3 ϵ and soluble monoclonal antibodies directed against CD28 (Pharmingen, San Diego, CA, USA). Briefly, immobilisation of anti-CD3 ϵ monoclonal antibodies (Pharmingen clone 2C11) onto plastic was performed by diluting the antibody to a final concentration of 5 μ g/ml in PBS and dispensing 30 μ l of the diluted monoclonal per well of a tissue culture treated flat bottom 96 well plate (Costar). The sealed plates were incubated for 16 hours at 4°C. Wells were washed three times with ice cold PBS to remove excess unbound antibody. BB-3103 inhibitor was added at a range of concentrations (0.02 μ M, 0.2 μ M, 2 μ M and 20 μ M final) to appropriate wells before addition of cells. Purified cells were plated out at 2.5x10⁵ per well in complete DMEM (Sigma) for murine cells or complete RPMI 1640 (Sigma) for human cells. Anti-CD28 (Pharmingen clone 37.51) monoclonal antibodies were added to the wells at a final concentration of 5 μ g/ml diluted in the appropriate cell culture medium in a final culture volume of 200 μ l. Addition of other exogenous soluble monoclonal antibodies was performed prior to incubation at 37°C in a humidified incubator with 5% CO₂ for 72 hours. Proliferation was assessed by incorporation of tritiated thymidine for 6 or 16 hours (as described in the text) and assayed by scintillation as described previously. All samples were tested in triplicate or sextuplet and each experiment repeated three times unless otherwise stated in the Figure legends.

2.2.3 CELL COUNTS

Cell counts from proliferation assays were performed on the days 1, 2 and 3 using haemocytometers and 0.4% trypan blue (Sigma) exclusion. 5×10^5 cells were dispensed per well of a tissue culture treated 96 well flat bottom plate in complete DMEM (Sigma), previously coated with excess anti-CD3 antibody (Pharmingen clone 2C11) as described above. $20 \mu\text{M}$ BB-3103 was added to appropriate wells and $5 \mu\text{g/ml}$ anti-CD28 mAb (Pharmingen clone 37.51) was also added to the culture for a final volume of $200 \mu\text{l}$ per well. Counts from each sample were performed in triplicate. The experiments were repeated in triplicate.

2.2.4 APOPTOSIS ASSAY (ANNEXIN-V-FITC STAINING)

Annexin-V-FITC staining of CD4⁺ cells was performed using an apoptosis detection kit following the manufacturer's instructions (BenderMed Systems, Vienna, Austria). Briefly, 5×10^5 proliferating cells (activated with anti-CD3 and anti CD28, as described in section 2.2.3) were centrifuged at $300 \times g$, 4°C and resuspended in $100 \mu\text{l}$ 1X calcium buffer provided in the kit. $5 \mu\text{l}$ Annexin-V-FITC conjugate was added to the resuspended cells and incubated on ice for 30 minutes. Cells were washed once in 1ml 1X ice cold calcium buffer and resuspended in $200 \mu\text{l}$ 1X calcium buffer containing $5 \mu\text{l}$ propidium iodide. Cells were incubated on ice for a further 10 minutes prior to flow cytometric analysis using Becton Dickinson FACSCalibur (BDIS, San Jose, CA, USA).

2.2.5 FLOW CYTOMETRY

All flow cytometry was performed on a Becton Dickinson FACSCalibur (BDIS, San Jose, CA, USA) and experiments were repeated three times. Phenotypic analysis of murine cell surface markers was accomplished using Pharmingen (Pharmingen, San Diego, CA, USA) monoclonal antibodies recognising the following cell surface proteins (CD4, CD8a, CD25,

CD27, CD30, CD40L, CD62L, CD69, CD95, CD95L, CD137). Appropriate isotype controls were also purchased from Pharmingen. Briefly, cells of interest were washed in ice-cold PBS and resuspended in FACS staining buffer at a final concentration of 10^6 cells per 100 μ l. 1 μ g of relevant monoclonal antibody was added per 10^6 cells and incubated in the dark on ice for 30 minutes. Samples were washed three times with 500 μ l ice-cold PBS and resuspended in ice-cold FACS fixing solution. Fixed samples were kept at 4°C and analysed as specified by Becton Dickinson.

2.2.6 ENZYME LINKED IMMUNOSORBANT ASSAYS (ELISAs)

Cytokine secretion was analysed by detecting soluble protein in aliquots of supernatants collected from cell cultures at 48hrs. Similar methods were used for murine and human cytokine detection. Reagents for cytokine detection and standards were purchased from Pharmingen (San Diego, CA, USA). Briefly, anti-cytokine capture antibodies were diluted to 1 μ g/ml in ELISA binding buffer. 50 μ l of diluted capture antibody was added to the wells of enhanced protein binding ELISA plates (Corning, NY, USA). The plates were sealed and incubated overnight at 4°C. Excess capture antibody was removed, 200 μ l of ELISA blocking buffer was added to each well and the plates incubated at 37°C for one hour. Wells were washed 3 times with ELISA wash buffer and 100 μ l of standards and samples were added to the wells (diluted in ELISA wash buffer). Plates were sealed and incubated overnight at 4°C. Wells were washed 4 times with 200 μ l ELISA wash buffer and 100 μ l of 1 μ g/ml biotinylated detection antibody diluted in ELISA wash buffer was added to each well. Plates were incubated for one hour at room temperature after which wells were washed 5 times with 200 μ l ELISA wash buffer and 200 μ l of 1 in 2000 diluted (manufacturer's instructions) Streptavidin-alkaline phosphatase conjugate (Amersham Life Sciences, Amersham, U.K.) in ELISA wash buffer was added to the wells and incubated for 30 minutes at room temperature. Wells were washed 7 times with ELISA wash buffer to remove excess alkaline

phosphatase and 200µl of ELISA substrate solution was added to wells. Plates were placed in the dark for colour change to occur and assayed at 10 minute intervals on a Microplate Reader 450 (BioRad Laboratories, Hemel Hempstead, U.K.) and associated software.

2.3 SIGNALLING

2.3.1 IMMUNOPRECIPITATION

In order to immunoprecipitate and detect proteins by western blotting, the activation method of CD4⁺ cells was modified. Each sample required 10⁷ purified cells which were stimulated in larger bulk cultures. Briefly, per sample, 10⁷ purified CD4⁺ T cells (resuspended in 1ml complete DMEM (Sigma)) were added to one well of tissue culture treated 24 well plates (Costar), previously coated with excess (20µg/ml) anti-CD3ε monoclonal antibody for 16 hours, as previously described. Plates were very briefly centrifuged at room temperature (100 x g for 15 seconds) to initiate contact between cells and immobilised monoclonal antibodies. Time points started once the plates were removed from the centrifuge. At appropriate time points, lysis of cells was performed by addition of 1ml 2 X Tris-based lysis buffer to each sample followed by thorough pipetting to ensure complete lysis. Lysed samples were transferred to fresh tubes and placed on ice for 30 minutes with vigorous vortexing at regular intervals. Samples were clarified by centrifugation at 14000xg at 4°C. Insoluble nuclei formed a pellet which was avoided as the supernatant was removed. Agarose conjugated monoclonal antibodies directed against tyrosine phosphorylated residues, PY-20 and PY-99 (Santa Cruz Biotechnology, Santa Cruz, USA– both antibodies mouse IgG_{2b} isotype) were added as supplied by the manufacturer (50µg antibody per 10⁷ cells) to the clarified supernatants and gently mixed at 4°C overnight. Samples were washed 5 times with 1ml freshly prepared ice-cold 1 X Tris based lysis buffer with 4°C 1000xg centrifugations. 50µl boiling Laemlli buffer (Sigma, Dorset, U.K) was added to each pellet

and samples were boiled for 5 minutes prior to immunoblotting. Positive control samples consisting of 5×10^6 unstimulated Jurkat cell pellets were lysed by addition of 20 μ l boiling Laemlli buffer.

2.3.2 IMMUNOBLOTTING

8% mini SDS-poly acrylamide gel electrophoresis (PAGE) gels were cast with 4% stacking gels on BioRad mini Protean II rigs (BioRad). Samples were electrophoresed at 200 Volts using SDS-Tris-Glycine electrophoresis buffer for one hour beside pre-stained molecular weight markers (Gibco BRL, Paisley, U.K.). Proteins were transferred to nitrocellulose (Amersham Pharmacia Biotech, Amersham, U.K.) using methanol based transfer buffer at 400 milliAmps for 45 minutes. Non-specific protein binding sites on the nitrocellulose membrane were blocked by incubation of the membranes in 50mls blocking buffer for one hour at 37°C or overnight at 4°C. Membranes were washed 3 times in 50 ml nitrocellulose wash buffer for 5 minutes. Appropriate primary antibodies were diluted in 20mls nitrocellulose wash buffer and incubated for one hour at 37°C with constant shaking. Antibodies used were mouse IgG₁ monoclonal anti-p56^{lck} (Pharmingen, San Diego, USA), mouse monoclonal IgG₁ anti-CD3 ζ chain (Santa Cruz Biotechnology, Santa Cruz, USA) and rabbit polyclonal IgG anti ZAP-70 (Santa Cruz Biotechnology, Santa Cruz, USA) which bind proteins from both murine and human species. Membranes were washed 5 times with 50mls nitrocellulose wash buffer at room temperature. Detection horseradish peroxidase (HRP) conjugated antibodies (Dako Corporation, Carpinteria, CA, USA; (a) HRP-Goat anti mouse Ig, or (b) HRP-Goat anti rabbit Ig) were diluted 1 in 2000 (manufacturer's instructions) in nitrocellulose wash buffer and incubated with the membranes for one hour at room temperature with gentle shaking. Membranes were washed a further 5 times with 50mls nitrocellulose wash buffer before 5 minute incubation with enhanced chemiluminescent

(ECL) reagent (Amersham Pharmacia Biotech, Amersham, U.K.). Excess ECL reagent was removed and the membrane placed under BioMax MS-1 X-ray sensitive film. Films were processed through an X-ray developer (X-Ograph Imaging Systems, Wilts, U.K.) at various exposure time points (1min, 3min, 5 min and 10 minutes). Optimal exposure time films were photographed using a white trans-illuminator and gel documentation system (Ultra Violet Products, Cambridge, U.K.).

2.3.3 CALCIUM DETECTION IN HUMAN CD4⁺ T CELLS

Calcium flux detection was performed using a modified method previously described (O'Flaherty and Rossi 1993) which examined $[Ca^{2+}]$ in neutrophils. Briefly, single cell suspension of human CD4⁺ T cells were isolated from PBMCs as previously described. Purified CD4⁺ T cells were resuspended in Hanks Balanced Salt Solution without Ca^{2+} or Mg^{2+} (Sigma) at a concentration of 10^7 cells/ml. $1\mu M$ Fura-2AM (Sigma) final concentration was added to the cells and incubated, shaking in the dark at $37^\circ C$ for 20 minutes. Cells were washed 3 times with HBSS (Sigma), resuspended in $37^\circ C$ HBSS (Sigma) and allowed to rest for 10 minutes at $37^\circ C$ to allow total de-esterification of Fura-2AM. Cells were centrifuged gently and resuspended in PBS giving a cell concentration of 4×10^6 /ml. 10^7 cells (2.5mls) were placed in a $37^\circ C$ heated, stirred cuvette fluorimeter (SPEX Fluoromax) with dual wavelength excitation (340 and 380 nm), emission at 510nm. After a 2 minute warm-up period in the heated chamber, stimulus in the form of monoclonal antibodies purchased from Pharmingen (anti-CD3 ϵ (HIT3a) or anti-CD62L (Dreg 56)) was added to give final concentrations as described in the figure legends. $[Ca^{2+}]$ variations were compiled over 10 minute periods and analysed using the following formula: Maximal (R_{max}) and minimum (R_{min}) fluorescence were determined by addition of $50\mu M$ digitonin and $25mM$ EGTA to samples respectively. $[Ca^{2+}]$ was calculated from the relationship:

$$[Ca^{2+}]_i = Kd \cdot (R - R_{min}) \div (R_{max} - R) \cdot \beta$$

where $[Ca^{2+}]_i$ is the cytosolic calcium concentration, R is the ratio of fluorescence obtained at 340 and 380nm, in the sample before calibration, and R_{max} is the fluorescence ratio under saturating $[Ca^{2+}]_i$, R_{min} is the fluorescence ratio in the absence of Ca^{2+} , K_d is the dissociation constant for Fura-2AM, taken as 224nm at 37°C and β is the fluorescence ratio at 340nm of cells in the absence and presence of Ca^{2+} .

2.4 CD4⁺ T CELLS SUBSETS

2.4.1 HUMAN CD4⁺ T CELL CLONE PROLIFERATION

Proliferation of two human CD4⁺ T cell clones (HA1.7 and AC1.1) was accomplished by purification of PBMCs from histocompatible donors (HLA-DRB1*1101 for HA1.7 and HLA-DQB1*0301 for AC1.1) as described previously. PBMCs were γ -irradiated with 2000rad using a ¹³⁷Caesium source. Irradiated PBMCs were resuspended in complete RPMI 1640 and plated out in tissue culture treated 96 round bottom well plates (Costar) at 2.5×10^4 cells/well. 1 μ g/ml final concentration appropriate peptide (HA 306-318 for HA1.7 and Der p 2 16-31 for AC1.1 kindly donated by Dr A Verhoef, Imperial College, London U.K.) was added to appropriate wells. Dilutions of BB-3103 (0.02, 0.2, 2 and 20 μ M) were added to appropriate wells. CD4⁺ T cell clones were washed three times in PBS, resuspended in complete RPMI 1640 and added to feeder cells at 2×10^4 cells/well in a final volume of 200 μ l. Cells were incubated at 37°C in a humidified incubator 5% CO₂ for 3 days. 50 μ l aliquots of supernatants were removed after 48hrs of culture for cytokine analysis. Proliferation was assessed by incorporation of tritiated thymidine for 16 hrs by scintillation as described previously. All samples were tested in triplicate.

2.4.2 PROLIFERATION OF PBMCs FROM ATOPIC AND NON-ATOPIC VOLUNTEERS

PBMCs isolated from atopic and non-atopic volunteers were plated out in tissue culture treated round bottom 96 well plates (Costar) at 2.5×10^5 cells/well in complete RPMI 1640 in a final volume of 200 μ l. Dilutions of BB-3103 were added to relevant wells. 10 μ g/ml (previously determined optimal concentration) of Der p 1 (in house purification) was added to appropriate wells. 50 μ l aliquots of supernatants were removed at 48 hrs. Plates were incubated at 37°C in a humidified incubator with 5% CO₂ for 3 days. Proliferation was assessed by incorporation of tritiated thymidine for 16 hrs as described before. Counts per minute (cpm) observed per sample were indicative of the extent of proliferation. All samples were tested in triplicate.

2.4.3 GENERATION OF MURINE Th₁ AND Th₂ TYPE CELLS

Naïve CD4⁺ T cells were enriched, resuspended in complete DMEM, plated out at 2.5×10^5 cells/well in tissue culture treated flat bottom 96 well plates (Costar) coated with immobilised anti murine CD3 ϵ monoclonal antibody (Pharmingen clone 2C11) as previously described. Anti-murine CD28 monoclonal antibody was added at a final concentration of 5 μ g/ml in complete DMEM. BB-3103 titrations were added to the appropriate wells. To generate a Th₁ type environment, 20ng/ml recombinant murine IFN γ (Serotech, Oxford, U.K.), 20ng/ml recombinant murine IL-12 (p70) (Pharmingen, San Diego, CA, USA) and 20 μ g/ml purified neutralising monoclonal anti IL-4 antibody (11B11 clone, in house purification) was added to the cultures. For a Th₂ type environment, 20ng/ml recombinant murine IL-4 (Serotech, Oxford, U.K.) and 20 μ g/ml purified neutralising monoclonal anti IFN γ antibody (HB170 clone, in house purification) was added to the cultures for a final volume of 200 μ l/well. Plates were

incubated at 37°C in a humidified incubator with 5% CO₂ for 3 days. Proliferation was assessed by incorporation of tritiated thymidine for 6 or 16 hrs as described in the figure legends. Counts per minute (cpm) observed per sample were indicative of the extent of proliferation. All samples were tested in triplicate and experiments performed three times unless otherwise stated in the text.

2.5 MATERIALS AND BUFFERS

All chemicals were purchased from Sigma (Dorset, U.K.) unless otherwise stated.

- **Red Blood Cell Lysis Buffer (Tris-NH₄Cl)**
0.017M Tris Base (T-1503) in 0.83% NH₄Cl(A-5666), pH 7.65 (HCl). Kept at 4°C.
- **Complete Murine DMEM culture media**
500mls DMEM (D-5921) with 10% foetal calf serum (FCS) (F-9665), 5x10⁻⁵ M β-mercaptoethanol (M-7522), 100IU/ml penicillin/streptomycin (Gibco, Paisley, U.K. 15070-071), 2mM L-Glutamine (Gibco, Paisley, U.K., 25033-010). Kept at 4°C.
- **Complete Human RPMI culture media**
500mls RPMI 1640 (R-0883) with 5% human AB serum [heat inactivated] (H-1513), 100IU/ml penicillin/streptomycin (Gibco, Paisley, U.K. 15070-071), 2mM L-Glutamine (Gibco, Paisley, U.K., 25033-010). Kept at 4°C.
- **MACS Buffer**
0.5% BSA (A-2153) in PBS⁻ (D-5652). Kept at 4°C.
- **FACS Staining Buffer**
10% normal mouse serum (Scottish Antibody Production Unit-SAPU, Scotland, U.K.-NMS) in PBS⁻ (D-5652). Kept at 4°C.
- **FACS Fixing Buffer**
2% paraformaldehyde (P-6148) in PBS⁻ (D-5652). Kept at 4°C.
- **ELISA Binding Buffer**
Carbonate-Bicarbonate buffer (C-3041). Made fresh.
- **ELISA Blocking Buffer**
PBS⁻ (D-5652) containing 5% Casein (C-7078) and 0.1% Tween-20 (P-7949). Made fresh.
- **ELISA Wash Buffer**
PBS⁻ (D-5652) containing 0.1% Tween-20 (P-7949). Kept at room temperature.
- **ELISA Substrate Solution**

E.L.I.S.A. ethanolamine buffer (Don Whitley Scientific, Shipton, U.K. E-016) containing 1mg/ml pNPP (N-2770). Made fresh.

- **1 X Tris-Based Cell Lysis Buffer**

50mM Tris (T-1503) pH7.4, 150mM NaCl (S-7653), 2mM EDTA (E-1644) pH 8.0, 5mM NaF (S-7920), 5mM Na₃VO₄ (S-6508), 1% NP-40/IGEPAL CA-630 (I-3021), anti-protease cocktail (P-2714). Made fresh. (2X concs doubled)

- **8% SDS-PAGE Gel (200mls)**

94.8mls dH₂O, 53.2mls 30% acrylamide/bis-acrylamide mix (A-3699), 50mls 1.5M Trizma Base pH 8.8 (T-1503), 1ml 10% SDS (L-4509). Kept at 4°C. Polymerisation of 5mls gel mixture catalysed by addition of 5µl TEMED (T-9281) and 50µl 10% APS (A-3678).

- **4% Stacking Gel (100mls)**

69.5% dH₂O, 17mls 30% acrylamide/bis-acrylamide mix (A-3699), 12.5mls 1.5M Trizma Base pH 8.8 (T-1503), 0.5ml 10% SDS (L-4509). Kept at 4°C. Polymerisation of 5mls gel mixture catalysed by addition of 5µl TEMED (T-9281) and 50µl 10% APS (A-3678).

- **SDS-Tris Glycine Electrophoresis Buffer**

25mM Trizma Base (T-1503), 250mM Glycine (G-7403), 0.1% SDS (L-4509). Kept at room temperature.

- **Methanol Based Transfer Buffer**

39mM Glycine (G-7403), 48mM Trizma Base (T-1503), 0.037% SDS (L-4509), 20% methanol (M-1770). Made fresh.

- **Blocking Buffer**

PBS⁻ (D-5652) with 0.1% Tween-20 (P-7949) and 5% BSA (A-2153). Made fresh.

- **Nitrocellulose Wash Buffer**

PBS⁻ (D-5652) with 0.1% Tween-20 (P-7949). Kept at room temperature.

- **Der p 1 purification**

Der p 1 group 1 allergen from *Dermatophagoides pteronyssinus* was purified by immunoaffinity chromatography as described before (Hoyne *et al.* 1993). Purity of protein was assessed electrophoresis on an staining 8% SDS-PAGE gel stained with Coomassie brilliant blue (B-5133) buffer and destain.

- **Neutralising antibody purification**

Neutralising antibodies to IFN γ and IL-4 were purified as described before (Darby *et al.* 1993) from B cell hybridoma supernatants (anti-IFN γ , HB170 clone; anti-IL-4, 11B11 clone). Purified antibodies were resuspended in PBS⁻(D-5652) and stored at 4°C.

- **Preparation of mitogens**

ConA (C-7275), PWM (L-9379) and LPS (L-2880) mitogens were resuspended at 1mg/ml in PBS⁻ and stored at -20°C. Anti- μ chain IgM was resuspended in PBS⁻ (D-5652) and stored at 4°C. PMA (P-8139) was resuspended in ethanol (E-7023) at 0.1mg/ml and stored at -20°C. Ionomycin (I-0634) was resuspended at 0.5mg/ml in ethanol (E-7023) and stored at -20°C.

- **Preparation of BB-3103 (BB-2116)**

BB-3103 was provided in powder form and resuspended at a final concentration of 20mM in DMSO (D-2650). The compound was stored at 4°C.

- **Coomassie Stain (1L)**

10% glacial acetic acid (A-6283), 40% methanol (M-1770) 1g Coomassie brilliant blue (B-5133).

- **Coomassie Destain**

10% glacial acetic acid (A-6283), 40% methanol (M-1770).

RESULTS AND DISCUSSIONS

All results are shown as means of three experiments unless otherwise stated in the text. Statistics were calculated using means of repeated experiments against a one-way ANOVA test. Significant p values are represented on graphs as follows $p < 0.05 = *$, $p < 0.01 = **$ and $p < 0.001 = ***$. Data points labeled 'Control' signify samples not containing BB-3103.

CHAPTER 3

3.1 PMA/IONOMYCIN INDUCED PROLIFERATION IS UNAFFECTED BY BB-3103

These experiments were designed to investigate if BB-3103 could affect the proliferation of T and B lymphocytes from spleens of naïve (unimmunised) C57BL/6 (H-2^b) mice stimulated in an antigen independent manner. PMA is a diacylglycerol (DAG) analogue which constitutively activates the mitogen activated protein kinase (MAPK) pathway (see section 1.6.5 and Figure 1.11), whereas ionomycin is a calcium ionophore which opens calcium channels and enables the influx of extracellular calcium ions into cells to activate calcineurin (see section 1.6.6 and Figure 1.11). Both pathways mentioned are required for full lymphocyte activation and proliferation (Watts and DeBenedette 1999). Splenocytes were isolated from C57BL/6 mice and cultured for 3 days in the presence of 0.5 µg/ml ionomycin and 0.1 µg/ml PMA. Proliferation was determined by thymidine incorporation for 6 hrs (Figure 3.1)

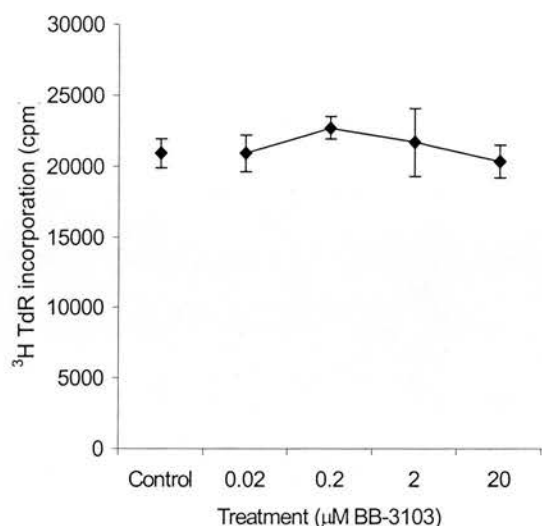


Figure 3. 1: C57BL/6 spleen proliferation to PMA/iono in the presence of BB-3103. Whole naïve C56BL/6 spleen isolated and cultured with 0.1μg/ml PMA and 0.5μg/ml ionomycin for 3 days in the presence of BB-3103 concentrations indicated on x-axis. Proliferation was determined by thymidine incorporation for the last 6 hours of culture. Graph represents mean proliferation of 4 animals. Background proliferation counts did not exceed 1000 cpm in all experiments.

BB-3103, over the concentrations tested, did not inhibit PMA/Ionomycin induced splenocyte proliferation. Since PMA and ionomycin bypass receptor mediated signalling, it appeared BB-3103 did not directly interfere with intracellular signalling events. BB-3103 is incapable of penetrating the cell and thus remains extracellular (Layton G., British Biotech, personal communication).

3.2 BB-3103 DOES NOT AFFECT B LYMPHOCYTE PROLIFERATION

In order to investigate the effect of BB-3103 hydroxamate inhibitor on the activation and subsequent proliferation of B lymphocytes, spleens from naïve C57BL/6 mice were cultured in the presence of the mitogen LPS (Figure 3.2), or activated by cross linking B cell receptor (BCR) with the use of anti- μ -chain of IgM F(ab')₂ monoclonal antibody (Figure 3.3) in the presence of various BB-3103 doses.

3.2.1 BB-3103 DOES NOT AFFECT LPS SPLENOCYTE PROLIFERATION

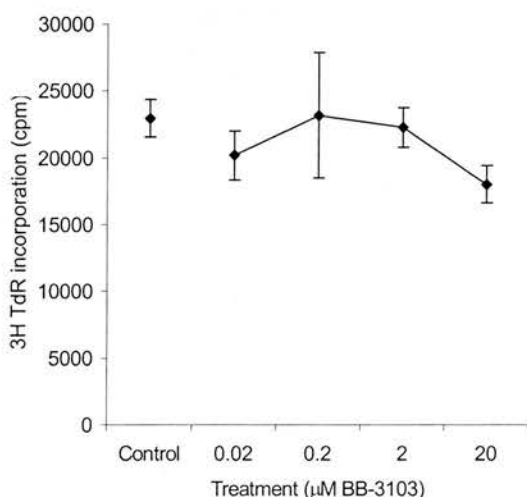


Figure 3. 2: C57BL/6 spleen proliferation to LPS in the presence of BB-3103. Spleen cells from naïve C57BL/6 mice cultured for 3 days in the presence of 5 μg/ml LPS and various doses of BB-3103 as indicated on the x-axis. Proliferation was determined by thymidine incorporation for the last 6 hours of culture. Graph represents mean of three animals. Background proliferation counts did not exceed 1000 cpm.

3.2.2 BB-3103 DOES NOT MODULATE BCR MEDIATED B CELL PROLIFERATION

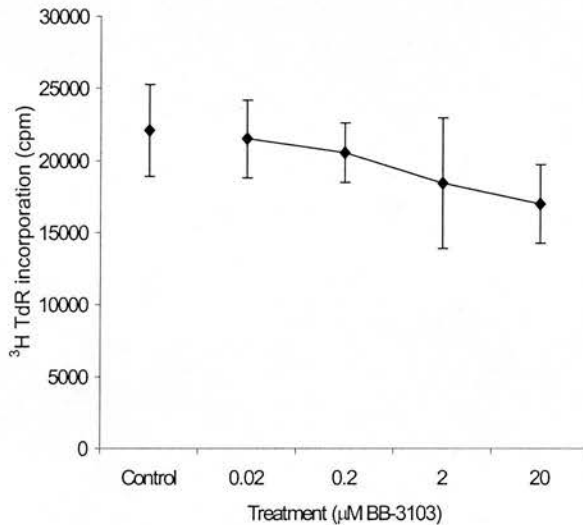


Figure 3. 3: Proliferation of B cells in C57BL/6 spleen by cross linking BCR in the presence of BB-3103. Spleen cells from naïve C57BL/6 mice cultured for 3 days in the presence of 20μg/ml anti μ-IgM F(ab')₂ and various doses of BB-3103 as indicated on the x-axis. Proliferation was determined by thymidine incorporation for the last 6 hours of culture. Graph represents means of three animals. Background proliferation did not exceed 1000 cpm.

Although no flow cytometry was performed on resulting cultures to establish B cell expansion, BB-3103 seemed to have no effect on cell activation and proliferation by different activation mechanisms investigated, LPS-CD14/TLR4 dependent activation (Hoshino *et al.* 1999) and anti-μ-IgM F(ab')₂-BCR dependent activation (Maruyama *et al.* 1985). Neither of these modes of cell activation were affected by the presence of metalloproteinase inhibitor suggesting MP activity may not essential for B cell activation and proliferation.

SUMMARY

- BB-3103 does not modulate splenocyte proliferation induced by:
 - PMA and Ionomycin
 - LPS

- Anti- μ -chain IgM

3.3 MITOGEN DRIVEN T LYMPHOCYTE PROLIFERATION IS DOWNREGULATED BY BB-3103

T lymphocytes can also be activated with mitogens. In these experiments, two mitogenic plant lectins were investigated, Concanavalin A (ConA) (Figure 3.4) and pokeweed mitogen (PWM)-(Figure 3.5). Lectin induced T cell stimulation is dependent on accessory cells which provided the necessary co-stimulatory signals to complete T cell activation (Torbett and Clark 1988). In these experiments, pre-determined optimal doses of each lectin were added to naïve spleen cell suspensions in the presence of BB-3103.

3.3.1 BB-3103 AFFECTS ConA MEDIATED T CELL PROLIFERATION

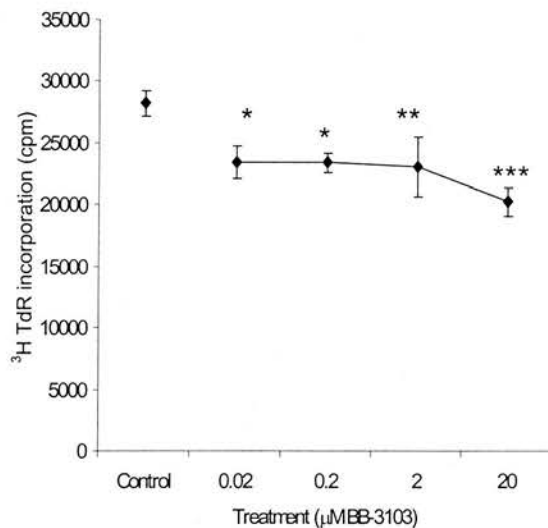


Figure 3. 4: C57BL/6 spleen proliferation to ConA in the presence of BB-3103. Naïve C56BL/6 spleen cells cultured with 2.5 μ g/ml ConA for 3 days in the presence of BB-3103 concentrations as indicated on the x-axis. Proliferation was determined by thymidine incorporation for the last 6 hours of culture. Graph represents means of three animals. Background proliferation did not exceed 1000 cpm.

BB-3103 downregulated splenocyte proliferation induced by ConA.

3.3.2 BB-3103 REDUCES PWM MEDIATED T CELL PROLIFERATION

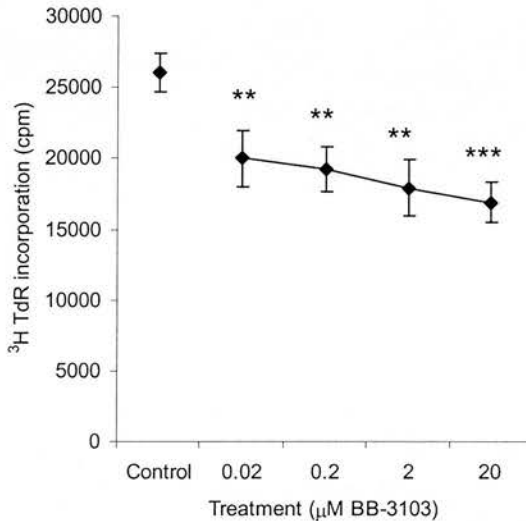


Figure 3. 5: C57BL/6 spleen proliferation to PWM in the presence of BB-3103. Naïve C56BL/6 spleen cells cultured with 1μg/ml PWM for 3 days in the presence of BB-3103 concentrations as indicated on the x-axis. Proliferation was determined by thymidine incorporation for the last 6 hours of culture. Graph represents means of three animals. Background proliferation did not exceed 1000 cpm.

It was observed that splenocyte proliferation induced with both plant lectin mitogens (ConA and PWM) was markedly inhibited by BB-3103 as compared to controls. These results suggested that the inhibition of metalloproteinase activity could downregulate T cell proliferation, since ConA and PWM are both known T cell activators. No flow cytometric analysis was performed on the resulting cultures however, therefore it can only be assumed the proliferation seen was due to T cell expansion.

SUMMARY

- BB-3103 reduced both ConA and PWM induced splenocyte proliferation by 30-35%

3.4 T LYMPHOCYTE PROLIFERATION IS AFFECTED BY BB-3103 IN THE ABSENCE OF ACCESSORY CELLS

The following series of experiments were designed to investigate the proliferation of various subsets of T lymphocytes in the absence of accessory cells in order to assess whether the inhibitory effect of BB-3103 was due to direct action on T lymphocytes or through costimulation provided by accessory cells.

Thy1 antigen (CD90) is expressed on murine CD4⁺ and CD8⁺ T lymphocytes (Gunter *et al.* 1984) and can be used as a cell surface molecule to isolate both cell types from other splenocytes using positive selection with magnetic associated cell separation columns (MACS, Miltenyi Biotech). Furthermore, CD4 and CD8 cell surface antigens may also be used to fractionate T cell populations (Schmitz *et al.* 1994) using the same positive selection mechanism.

In order to investigate the proliferation of purified T cell subsets in the absence of accessory cells, TCRs were cross-linked using immobilised anti-CD3 ϵ mAb and costimulation provided by 5 μ g/ml soluble anti-CD28 mAb. The actual amounts of anti-CD3 ϵ which immobilised to the plate was not assayed, but presumed to be similar between each experiment.

3.4.1 BB-3103 INHIBITS THE PROLIFERATION OF PURIFIED CD90⁺ CELLS

MACS enriched CD90⁺ cells were activated for 3 days using immobilised anti-CD3 mAb and soluble anti-CD28 mAb (5µg/ml) in the presence of BB-3103 (Figure 3.6 below).

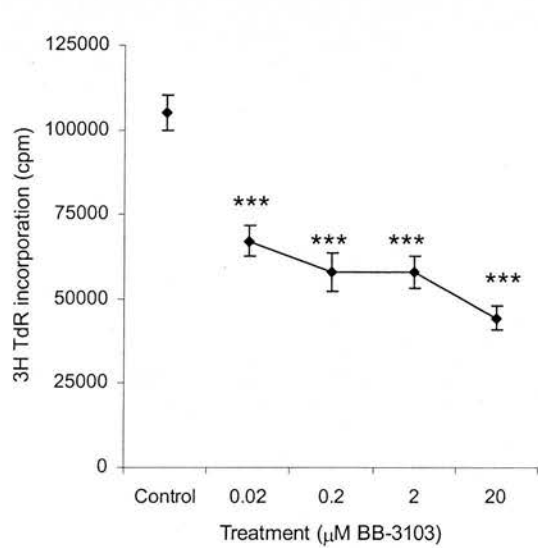


Figure 3. 6: Proliferation of purified CD90⁺ cells to anti-CD3 mAb & anti-CD28 mAb in the presence of BB-3103. Proliferation of CD90⁺ cells isolated from naïve C57BL/6 spleens and stimulated for 3 days with immobilised anti-CD3 mAb and 5µg/ml soluble anti-CD28 mAb in the presence of inhibitor as indicated on the x-axis. Proliferation was determined by thymidine incorporation for the last 6 hours of culture. Graph represents means of three animals. Background proliferation did not exceed 5000 cpm.

3.4.2 BB-3103 INHIBITS THE PROLIFERATION OF PURIFIED CD4⁺ CELLS

MACS enriched CD4⁺ T cells were activated for 3 days using immobilised anti-CD3 mAb and soluble anti-CD28 mAb (5 μ g/ml) in the presence of various doses of BB-3103 (Figure 3.7 below).

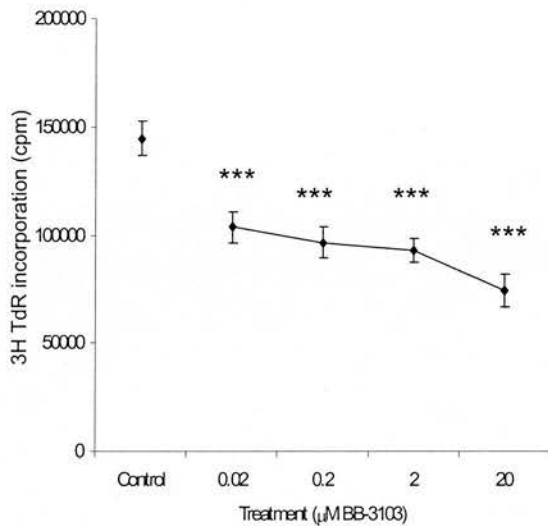


Figure 3. 7: Proliferation of purified CD4⁺ cells to anti-CD3 mAb and anti-CD28 mAb in the presence of BB-3103. Proliferation of purified CD4⁺ cells isolated from naïve C57BL/6 spleens and stimulated for 3 days with immobilised anti-CD3 mAb and 5 μ g/ml soluble anti-CD28 mAb in the presence of inhibitor as indicated on the x-axis. Proliferation was determined by thymidine incorporation for the last 6 hours of culture. Graph represents means of three animals. Background proliferation did not exceed 5000 cpm.

3.4.3 BB-3103 INHIBITS THE PROLIFERATION OF PURIFIED CD8⁺ CELLS

The effect of BB-3103 on CD8⁺ T lymphocytes was similarly investigated. Figure 3.8 illustrates the decrease in proliferation of enriched CD8⁺ T cells activated with immobilised anti-CD3 mAb and 5 µg/ml soluble anti-CD28 mAb in the presence of various doses of BB-3103.

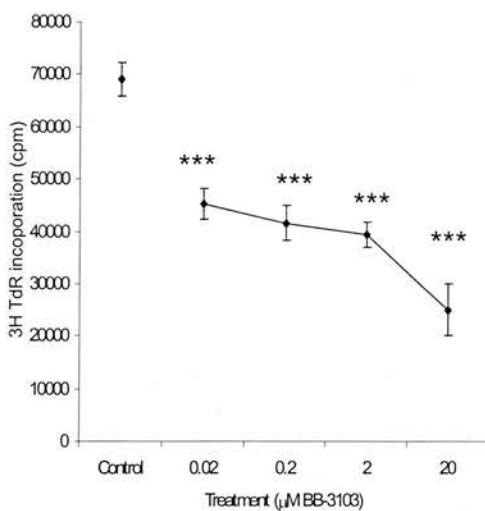


Figure 3. 8: Proliferation of purified CD8⁺ cells to anti-CD3 mAb and anti-CD28 mAb in the presence of BB-3103. Proliferation of CD8⁺ cells purified from naïve C57BL/6 spleens and stimulated for 3 days with immobilised anti-CD3 mAb and 5 µg/ml soluble anti-CD28 mAb in the presence of BB-3103 inhibitor as indicated on the x-axis. Proliferation was determined by thymidine incorporation for the last 6 hours of culture. Graph represents means of three animals. Background proliferation did not exceed 5000 cpm.

MACS enrichment of spleen cells led to 96% purity for CD4⁺, 89% for CD8⁺ and 91% for CD90⁺ cells populations. When these cells were stimulated *in vitro* with immobilised anti-CD3 mAb and soluble anti-CD28 mAb, strong proliferation was observed. However, consistent with the previous lymphocyte proliferation data observed using mitogenic lectins, addition of BB-3103 could downregulate T cell proliferation.

SUMMARY

- BB-3103 reduced proliferation of CD90⁺, CD4⁺ and CD8⁺ enriched lymphocytes by 58%, 50% and 64% respectively.
- Enriched T lymphocytes were used in these assays, therefore, these data suggest that BB-3103 acted directly on T cells.

DISCUSSION (CHAPTER 3)

It has not been previously reported that inhibition of metalloproteinase activity is capable of downregulating T lymphocyte proliferation.

The experiments described so far reveal that BB-3103 inhibits proliferation of enriched T lymphocytes but does not affect the proliferation of splenocytes to LPS or B cells through B cell receptor. These results are surprising, since, while this is the first demonstration that T lymphocytes require metalloproteinase activity for normal proliferation to occur, the same could not be demonstrated in B lymphocytes. The activation of B cells involved cross-linking the surface IgM (BCR) with the use of anti- μ IgM F(ab')₂ fragments which induces signalling events within the B cell similar to those observed in T lymphocytes (Harnett and Rigley 1992). This suggests that the mechanism by which metalloproteinase activity increases T cell proliferation is either not present, not required, or is redundant in B cells.

T lymphocyte proliferation was still inhibited when activated as a highly enriched population suggesting that the metalloproteinase responsible for T lymphocyte proliferation was not secreted by accessory cells which were present in previous, mitogen induced proliferation which investigated whole splenocytes.

Activation of splenocytes, however, with PMA and Ionomycin mitogens revealed that proliferation was not inhibited. These data indicated that the metalloproteinase substrate

responsible for fully activating T lymphocytes may act upstream of PLC γ phosphorylation and activation (see Figure 1.11). It is possible however, that the optimal mitogenic doses used in these experiments could have clouded any other, downstream effects which could have been modulated by BB-3103.

Another interesting observation is that stimulation of TCR with anti-CD3 mAb and anti-CD28 mAb activated different pathways to those activated through mitogenic lectins. The pathways leading from TCR activation are well established (Cantrell 1996), whereas, the mitogenic characteristic of lectins is poorly understood. Concanavalin A, for example, is thought to induce Na⁺/H⁺ exchange in T lymphocytes (Grinstein *et al.* 1987) which subsequently leads to IL-2 production. It is evident, however, that optimal T lymphocyte proliferation, whether through TCR or mitogenic plant lectins, utilises metalloproteinase activity, whereas, BCR or LPS mediated B cell activation is not dependent on metalloproteinase activity.

The proliferation assays performed in this Chapter are, however, limited in as far as kinetics of BB-3103 effects are concerned. This issue is highlighted later in the thesis (Fig 4.1) where cells treated with BB-3103 demonstrated a delayed proliferation profile rather than a complete inhibition. To address this issue, experiments investigating proliferation at various time points (24, 48, 72, 92 and 120 hrs) should have been performed in order to establish the kinetics of cell proliferation in the presence of BB-3103. The experiment shown in Fig 4.1 in fact suggests that cell treated with BB-3103 are not inhibited, but delayed in their proliferation, thus suggesting that the results shown in this Chapter are only investigating a small window of a kinetic response, not showing the real effects of BB-3103. Another issue which should have been investigated is the effect of BB-3103 addition at different time points using these stimulation methods, this would resolve whether BB-3103 could downregulate thymidine incorporation when added after stimulation rather than at the same time. The

experiments shown in Chapter 3 are also incomplete in as far as flow cytometry was never performed on the samples prior to thymidine incorporation. It is impossible to assess which cell types were thus affected by BB-3103 where splenocytes were stimulated with mitogenic lectins (ConA and LPS). Although the general understanding is that LPS activates B cells, this was not checked in these experiments and it can only thus be *assumed* that BB-3103 does not affect B cell proliferation. Similarly, Concanavalin A is a known T cell stimulator, however, since flow cytometric analysis was not performed on cells stimulated with ConA, the results can only be *interpreted* as BB-3103 affecting T cell proliferation. In experiments where enriched populations of T cells were used, the thymidine incorporation could only have been provided from the starting population and therefore are more indicative that BB-3103 only affects T cells. There are also, however, some limitations to these experiments since only optimal doses of immobilised anti CD3 and CD28 mAb were used. It was not considered whether at sub-optimal or supra-optimal antibody doses, BB-3103 would have the same effect. Similarly, varying the doses of lectins may have generated different patterns of inhibition, if any. Another issue which was not investigated was the fact that BB-3103 irreversibly binds in a 1:1 ratio with metalloproteases therefore it is conceivable that over time, the actual concentration of BB-3103 within the culture was reducing over time. To address this issue, proliferation experiments where adding a constant amount of BB-3103 on a daily basis should have been performed. These are all issues which could have generated a better understanding of BB-3103 kinetics and which cells were affected by BB-3103.

The percentage of proliferation inhibition generated from these experiments appears not to be consistent throughout the thesis and this may be attributed to either a slow degradation of BB-3103 through time and also a different batch of BB-3103 provided by British Biotech plc.

CHAPTER 4

4. THE INHIBITION OF T LYMPHOCYTE PROLIFERATION IS NOT DUE TO APOPTOSIS OR TOXICITY

The reduction in proliferation of T lymphocytes observed in the previous experiments was not due to toxic effects of BB-3103. This is supported by the finding that PMA/Ionomycin stimulation of whole splenocytes was not affected by BB-3103 when used at the same molarity as the TCR mediated proliferation experiments.

In order to assess apoptosis and/or toxicity of BB-3103 on T lymphocytes, cell counts were performed during a 3 day immobilised anti-CD3 mAb and 5µg/ml soluble anti-CD28 mAb proliferation assay (Figure 4.1). This was followed by FACS analysis looking for membrane bound AnnexinV-FITC conjugate (Figure 4.2) which binds phospholipid phosphatidylserine (PS). PS is translocated from the inner leaflet of the plasma membrane to the outer leaflet during very early phases of apoptosis and can thus bind AnnexinV-FITC extracellularly and can be used to detect early indications of programmed cell death by flow cytometry.

Propidium Iodide was used in conjunction with AnnexinV-FITC to detect membrane integrity of the cells, thus distinguishing necrotic cells and late apoptosis.

4.1 BB-3103 INHIBITS EARLY CD4⁺ T CELL EXPANSION

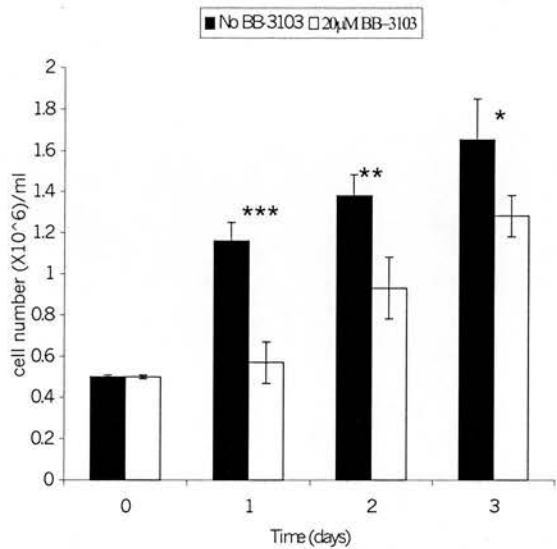


Figure 4. 1: BB-3103 affects cell number in proliferation assays. Cell counts of purified CD4⁺ T cells activated using immobilised anti-CD3 mAb and 5µg/ml soluble anti-CD28 mAb in the presence of 20µM BB-3103 over a period of 3 days. The p symbols (*) represent ANOVA statistics between the two samples within each daily group. Each sample was counted in triplicate. The graph represents the mean of three animals.

The cell number observed at each time point in the presence of BB-3103 was significantly lower than in the absence of BB-3103. An interesting observation drawn from these experiments is that the cell number in BB-3103 treated groups, although still lower than control groups, started to increase on day 2. The major inhibition in proliferation was seen on day 1 suggesting that BB-3103 affected one or more early events in the T cell activation and clonal expansion and enhances the issue of kinetics of proliferation in the presence of BB0-3103 (see Discussion Chapter 3).

4.2 PRESENCE OF BB-3103 DOES NOT INCREASE APOPTOSIS

Figure 4.2 shows that there is no increased apoptosis in BB-3103 treated groups. Cells in the lower left quadrants do not stain for either Annexin-V or PI and are, therefore, considered to be viable cells. Figure 4.2 panel A (positive control) demonstrates 42% of aged (16hrs in culture) neutrophils are actively undergoing early apoptosis (lower right quadrant-LR). These cells are staining positive for Annexin V-FITC indicating the cell membrane has lost symmetry and PS has translocated to the outer leaflet of the plasma membrane.

The upper right quadrants (UR) select cells at advanced stages of apoptosis, since these cells stain positive for Annexin-V and are also Propidium Iodide (PI) positive due to increased plasma membrane permeability.

Events acquired in the upper left quadrants (UL), are cells which stain positive for PI, but negative for AnnexinV-FITC due to the membranes having lost so much integrity they are no longer capable of binding Annexin-V.

There were no significant differences between cells treated in the absence of BB-3103 (Figure 4.2 panel B) and in the presence of 20 μ M BB-3103 (Figure 4.2 panel C) in any of the quadrants, suggesting the observed decrease in proliferation in the presence of BB-3103 was not due to increased apoptosis or necrosis.

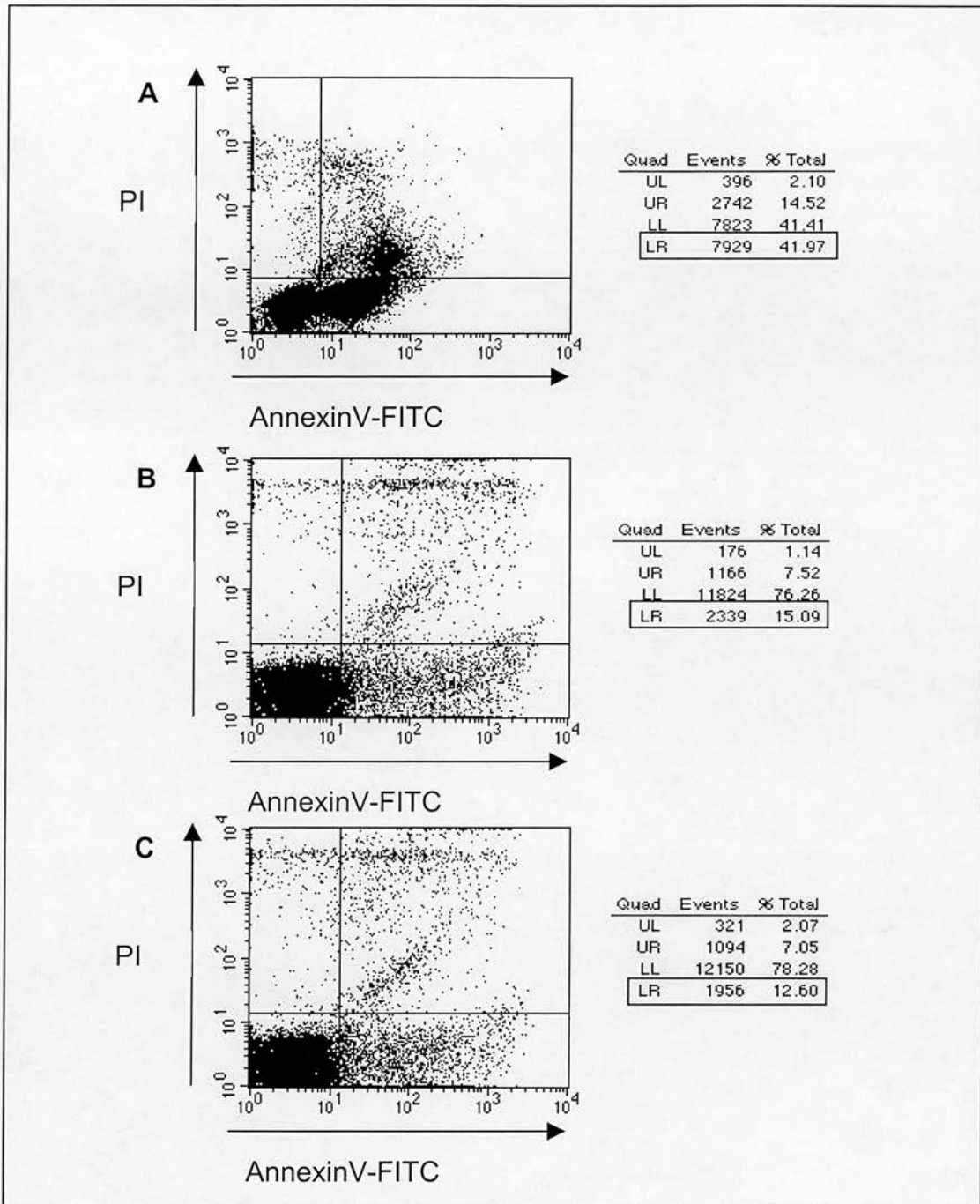


Figure 4. 2: BB-3103 does not increase Annexin-V-FITC staining on CD4⁺ T cells. FACS analyses of CD4⁺ T cells treated with BB-3103 do not show increased apoptosis. Panel A shows purified human neutrophils aged overnight at 37°C (+ve control). Panel B shows purified murine CD4⁺ T cells activated for 2 days in the absence of BB-3103 with immobilised anti-CD3 mAb and 5μg/ml soluble anti-CD28 mAb. Panel C shows similarly activated CD4⁺ T cells for 2 days in the presence of 20μM BB-3103. Values in boxes (lower right quadrant, or LR) are the values of positively stained Annexin-V cells undergoing early apoptosis. Cells in upper right quadrant (UR values) represent cells positively staining AnnexinV and PI denoting cells in a late stage of apoptosis.

SUMMARY

- BB-3103 does not induce apoptosis or necrosis in proliferating CD4⁺ T cells
- BB-3103 prevents increase in numbers of CD4⁺ T cells early in proliferation assays.

4.3 BB-3103 DOES NOT AFFECT IL-2 DEPENDENT AND INDEPENDENT TRANSFORMED T CELL LINES

The effect of BB-3103 inhibitor was assessed on two transformed T cell lines Jurkat E6.1, EL4 and the IL-2 dependent cell line, HT-2. Jurkat T cell line clone E6-1 was derived from an acute human T cell leukemia and consists of CD4⁺ T cells (Weiss *et al.* 1984). EL4 was established from a murine lymphoma (Shevach *et al.* 1972) and HT-2 is a murine T helper cell line which was originally established by limiting dilution in the presence of IL-2, and is highly dependent on IL-2 for proliferation (Woods *et al.* 1987). HT-2 cells are capable of growing in the presence of IL-2 alone without the requirement for TCR engagement or co-stimulatory signals. EL-4 and Jurkat cells proliferate continuously *in vitro* without the requirement of any stimulus. As shown in Figures 4.3 and 4.4, BB-3103 did not affect the proliferation of these cells even at the highest dose used of 20μM. Similarly, BB-3103 did not affect the proliferative response of HT-2 cells when cultured in the presence of various doses of IL-2. It would, therefore, appear that the IL-2 signalling pathway is not dependent on active metalloproteinases and the transformed status of Jurkat E6.1 and EL4 bypasses any requirement for metalloproteinase activity.

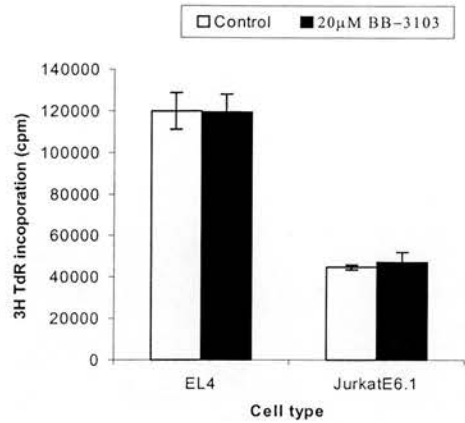


Figure 4. 3: Proliferation of EL4 and Jurkat E6.1 in the presence of 20µM BB-3103. Graph representing proliferation of transformed T cell lines in the presence or absence of 20µM BB-3103. Cells were cultured in the presence of 20µM BB-3103 without stimulus for 24hours. Thymidine incorporation was determined for the last 6 hours of culture. Graph represents mean of three experiments.

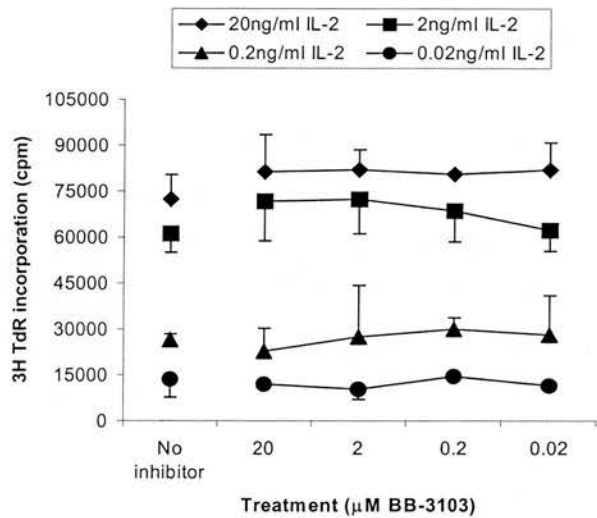


Figure 4. 4: Proliferation of HT-2 cells in the presence of BB-3103. HT-2 cells were grown in different concentrations of IL-2 as indicated in the legend. Titration of BB-3103 into each sample revealed that HT-2 cells were not affected by BB-3103. Thymidine incorporation was determined for the last 6 hours of culture. Graph represents mean of three experiments. Background proliferation (no IL-2) did not exceed 500cpm.

SUMMARY

- BB-3103 does not inhibit:
 - Proliferation of transformed T cell lines
 - IL-2 mediated proliferation of HT-2 cells.

4.4 IC₅₀ VALUES FOR BB-3103 IN T CELL PROLIFERATION

The inhibitory efficiency of pharmaceutical compounds is measured in IC₅₀ values, which are the concentrations of compounds at which the activity of what the compound is being tested against, is reduced by half regardless of cytotoxicity, in cellular systems. For these experiments purified CD4⁺ T cells were isolated from C57BL/6 spleens and activated using immobilised anti-CD3 mAb and soluble anti-CD28 (5µg/ml) mAb for 3 days in the presence of a much broader titration of inhibitor. The concentrations ranged from 2×10^{-5} to 2×10^3 µM BB-3103 (Figure 4.5 below)

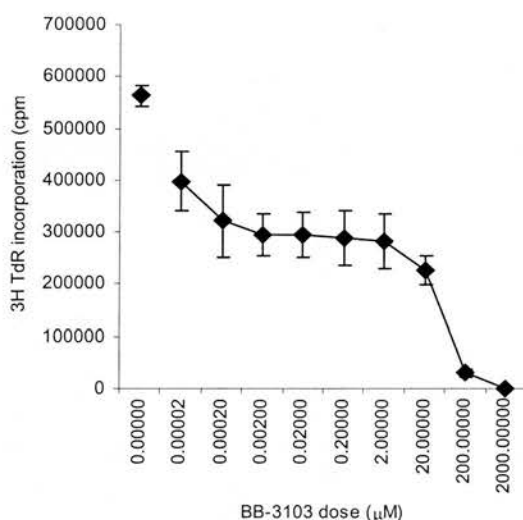


Figure 4. 5: IC₅₀ curve for BB-3103 in CD4⁺ T cells. Graphic representing the inhibition of CD4⁺ T cell proliferation in the presence of a large concentration range of BB-3103. The IC₅₀ values range from 0.0002µM to 2µM BB-3103. Graph represents mean of experiments performed on two animals. Thymidine incorporation was determined for the last 16 hours of culture. Background proliferation did not exceed 10000 cpm.

Establishing the exact IC₅₀ value for BB-3103 on T cell proliferation was extremely difficult since the inhibitory curve was bimodal and the 50% inhibition value lay on a plateau which encompassed 5 concentration values of BB-3103 (2×10^{-4} to 2µM BB-3103). The presence of the plateau seen in the inhibition curve indicated that BB-3103 was capable of inhibiting CD4⁺ T cell proliferation by half and the higher concentrations used (above 20µM) were

more than likely toxic due to elevated vehicle concentrations (DMSO) although this should have been investigated by titrating equivalent DMSO concentrations to proliferating CD4⁺ T cells.

SUMMARY

- IC₅₀ values for BB-3103 are broad and span concentrations from 2x10⁻⁴ to 2μM.

DISCUSSION (CHAPTER 4)

In the presence of BB-3103, T lymphocyte proliferation was inhibited. The experiments in this chapter were designed to determine whether the reduction in proliferation was due to cell apoptosis or necrosis. Assessing cell number by simply counting viable cells, revealed that 20μM BB-3103 was capable of inhibiting early cell division, since T lymphocyte division occurs every 6-8hrs (Cantrell 1996). Cell proliferation, however, recovered at later stages (3 days) but was still significantly lower than CD4⁺ T cells activated in the absence of BB-3103. These data suggested that the presence of BB-3103 either inhibited early events in activation of all T lymphocytes or that BB-3103 was incapable of inhibiting the proliferation of a minor population which, over time, expanded sufficiently to generate 30% less proliferation as compared to no inhibitor controls. As discussed at the end of Chapter 3, this 'recuperation' sheds some light onto the issue of kinetics studies to be performed. The data in Fig 4.1 highly suggests that had the experiment been taken further, the cell treated with BB-3103 may have demonstrated a *delayed* proliferation curve rather than an inhibition altogether.

AnnexinV binding to T lymphocytes did not increase in the presence of 20μM BB-3103, excluding the possibility of reduced counts being attributed to increased apoptosis. The

presence of PI in the assay investigated possible necrotic effects of BB-3103 but showed no increase in staining in the presence of BB-3103.

The concentration of inhibitor used in these apoptosis assays, 20 μ M, fell outwith the IC₅₀ range but still demonstrated no necrotic or apoptosis inducing properties on activated CD4⁺ T cells. Investigating the IC₅₀ values of BB-3103 on CD4⁺ T lymphocyte proliferation demonstrated a sudden reduction in proliferation between 200 and 2000 μ M concentrations. This may have been due to cytotoxic effect of DMSO which exceeded 0.05% at those concentrations. Exceeding 0.05% DMSO in the absence of BB-3103 downregulated T cell proliferation (results not shown and Dr R Rintoul, personal communication). To complete this experiment, a second titration of vehicle only (in this case, DMSO) should have been performed in order to establish whether the toxicity could be attributed to DMSO or whether this was entirely a BB-3103 cytotoxicity effect. The thymidine incorporation in these assays is noticeably higher than in other experiments and this cannot be attributed to any known factor other than a 16hr thymidine incorporation uptake period.

Two transformed T cell lines, Jurkats and EL-4, were grown in the presence of BB-3103 and revealed no change in cell expansion, suggesting that only TCR driven proliferation of naïve CD4⁺ T cells was capable of being inhibited, and the mechanism by which BB-3103 could inhibit proliferation was redundant or not present in transformed T cells.

BB-3103 did not affect the IL-2 driven proliferation response in HT-2 cells. These data strongly suggested that BB-3103 could not interfere with the IL-2 JAK/STAT signalling pathway (Beadling *et al.* 1994), although HT-2 cells were originally isolated and grown under highly selective and abnormal circumstances (Woods *et al.* 1987).

Taken together, the data presented so far, support the idea that, in the presence of BB-3103, T cells cannot be activated maximally because of absent metalloproteolytic activity. Also, since BB-3103 did not affect the IL-2 driven proliferation of HT2 cells, the observed decrease in CD4⁺ T cell proliferation could be due to decreased IL-2 production in the presence of inhibitor.

CHAPTER 5

5.1 BB-3103 REDUCES IL-2 SECRETION IN ACTIVATED CD4⁺ T CELLS

Since proliferation of activated CD4⁺ T cells was reduced in the presence of BB-3103, the cytokine secretion patterns were analysed. CD4⁺ T cells purified from spleens of unimmunised animals were activated with immobilised anti-CD3 mAb and 5µg/ml soluble anti-CD28 mAb for 48hrs in the presence or absence of BB-3103 as indicated on the x-axis (Figure 5.1).

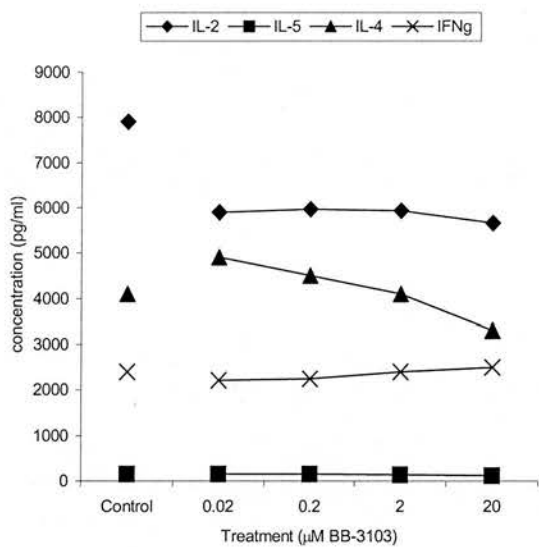


Figure 5. 1: Cytokine secretion of activated CD4⁺ T cells in the presence of BB-3103: Graph representing IL-2, IL-5, IL-4 and IFN γ concentrations in supernatants of activated CD4⁺ T cells at 48hrs. IL-2 concentration was reduced by approximately 25% in the presence of BB-3103, whereas other cytokines analysed showed no variation in the presence of inhibitor. Graph represents mean of two animals. IL-5 concentrations fell beneath detection range and therefore may not accurately represent actual IL-5 concentrations in the culture medium.

IL-2 secretion was reduced by 25% in the presence of inhibitor suggesting that this decrease may be one factor in the observed decrease in CD4⁺ T cell proliferation.

5.2 PHENOTYPIC ANALYSIS OF CD4⁺ T CELLS IN THE PRESENCE OF BB-3103

Since BB-3103 inhibited CD4⁺ T cell proliferation it was of interest to investigate the effect of BB-3103 on surface markers of CD4⁺ T cells. Flow cytometric analysis was performed on purified, activated cells (immobilised anti-CD3 mAb and 5µg/ml soluble anti-CD28 mAb) in the presence or absence of 20µM BB-3103 for the following cell surface proteins:- CD25, CD69, CD40L, Fas, FasL, CD62L, CD27, CD30, 4-1BB/CD137 (Table 5.1 below). The mean fluorescence intensity values (MFI) indicated in Table 5.1 are not representative of poisson distribution, and represents total fluorescence of cell populations which may contain 2 or more fluorescence intensities (see Fig. 5.2 for example). The use of MFI in this thesis is used as a mean of interpreting general effects by BB-3103 rather than describing poisson distribution of staining patterns.

	day 0	day 1		day 2		day 3	
	WT	control	20uM BB	control	20uM BB	control	20uM BB
CD25	6.08	142.41	113.4	113.33	185.3	238.82	186.24
CD69	10.76	1163	1033.87	1144.29	1083.51	913	915
CD40L	8.45	8.58	11.11	10.93	17.91	21.43	33.15
Fas	97.59	162	144	265.2	238.62	327.08	364.51
FasL	9.29	17.74	17.28	15.47	13.95	17.31	18.93
CD62L	90.02	39.34	261.15	50.58	235.13	47.92	173.69
CD27	291.18	155.84	375.38	324.89	534.12	326.05	664.62
CD30	11.24	29.27	36.59	47.04	42.93	77	98.84
4-1BB	8.41	42.59	37.56	57.06	71.52	134.74	118.5

Table 5. 1: Summary of the phenotypic data acquired by flow cytometry on purified CD4⁺ T cells activated with immobilised anti-CD3 mAb and 5µg/ml soluble anti-CD28 mAb in the presence or absence of 20µM BB-3103. The numbers indicate mean fluorescent intensity values found for each marker (MFI). Results show that CD27 and CD62L expressions are highly upregulated in the presence of BB-3103. Results for shaded cell surface proteins are discussed in more detail later in the text. Values indicated in the table are representative of triplicates. See Figs 5.2-5.5 for histogram profiles.

5.2.1 BB-3103 AFFECTS CD27 EXPRESSION ON CD4⁺ T CELLS

Of the markers tested, significant inhibition of activation induced downregulation was observed for CD27 (Table 5.1 and Figure 5.2 below)

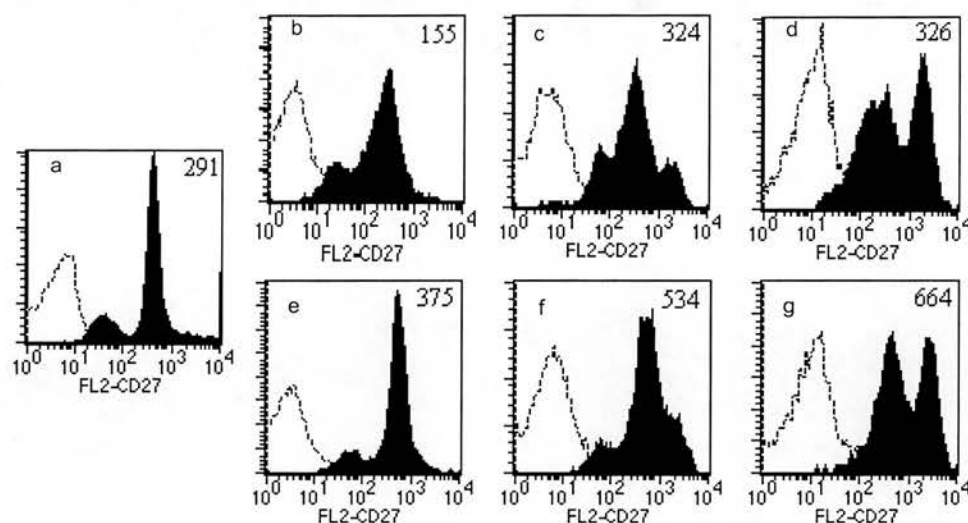


Figure 5. 2: BB-3103 affects CD27 expression on CD4⁺ T cells. Analysis of CD27 expression on naïve C57BL/6 enriched CD4⁺ T cells activated with immobilised anti-CD3 mAb and soluble anti-CD28 mAb over a period of 3 days in the presence or absence of BB-3103. Panels represent: a) freshly purified CD4⁺ T cells, b) activated CD4⁺ T cells for 24hrs, c) activated CD4⁺ T cells for 48hrs, d) activated CD4⁺ T cells for 72hrs, e) activated CD4⁺ T cells for 24hrs+20 μ M BB-3103, f) activated CD4⁺ T cells for 48hrs+20 μ M BB-3103, g) activated CD4⁺ T cells for 72hrs+20 μ M BB-3103. Dotted lines represent isotype controls, filled profiles represent positively stained cell counts. MFI values for CD27 expression are indicated top right in each panel. The histograms shown are from one experiment and are representative of three animals.

From the results presented above, CD27 was expressed at high levels on the surface of resting naïve CD4⁺ T cells, supporting published data (Hintzen *et al.* 1993). CD27 was, however, downregulated following activation at 24hrs but by day 3, a population of CD27^{hi} CD4⁺ T cells was observed. Presence of BB-3103 during the activation process inhibited

early CD27 downregulation events and maintained high CD27 expression throughout the 3 days. Thus, it appeared that CD27 may be shed by a metalloproteinase enzyme which could have been inhibited by the presence of hydroxamate containing pseudopeptide, BB-3103, although the shedding process was never examined by soluble CD27 ELISA of culture supernatants.

5.2.2 CD62L EXPRESSION ON CD4⁺ T CELLS IS MAINTAINED IN THE PRESENCE OF BB-3103

It has also been reported that CD62L is shed from the surface of a variety of immune cells upon activation (Kuhns *et al.* 1995; Matsumoto *et al.* 1998). The addition of BB-3103 to activated CD4⁺ T cells inhibited CD62L downregulation throughout the 3 days of the assay (Figure 5.3).

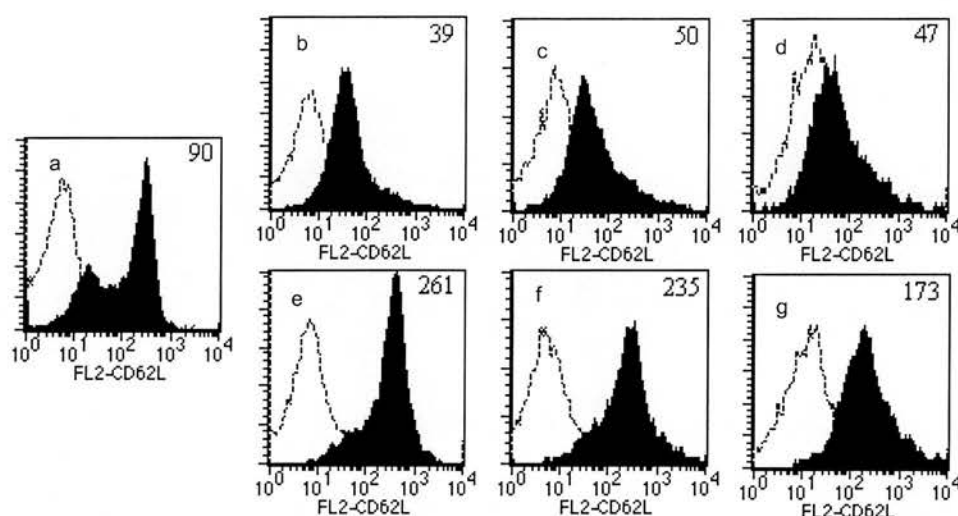


Figure 5. 3: BB-3103 affects CD62L expression on wild type (WT) CD4⁺ T cells. Analysis of CD62L expression on naïve C57BL/6 enriched CD4⁺ T cells activated with immobilised anti-CD3 mAb and soluble anti-CD28 mAb over a period of 3 days in the presence or absence of BB-3103. Panels represent: a) freshly purified CD4⁺ T cells, b) activated CD4⁺ T cells for 24hrs, c) activated CD4⁺ T cells for 48hrs, d) activated CD4⁺ T cells for 72hrs, e) activated CD4⁺ T cells for 24hrs+20μM BB-3103, f) activated CD4⁺ T cells for 48hrs+20μM BB-3103, g) activated CD4⁺ T cells for 72hrs+20μM BB-3103. Dotted lines represent isotype controls, filled profiles represent positively stained cell counts expressing CD62L. MFI values for CD62L expression are indicated top right in each panel. The histograms shown are from one experiment and are representative of three animals.

CD62L was downregulated from the surface of the CD4⁺ T cells upon activation (Figure 5.3). The presence of BB-3103 during the activation process inhibited downregulation, suggesting the process may have been mediated by a metalloproteinase. The decrease in CD62L

expression in BB-3103 treated groups over time may have been due to decrease in molarity of free inhibitor in the culture medium since one molecule of pseudopeptide irreversibly binds and inhibits one molecule of metalloproteinase.

SUMMARY

- CD27 may have been downregulated by metalloproteinase activity upon CD4⁺ T cell activation since this process was inhibited by BB-3103. CD27 was subsequently upregulated in both BB-3103 and control samples.
- CD62L was downregulated upon activation possibly by metalloproteinase activity since this process was inhibited by BB-3103.

5.2.3 BB-3103 DOES NOT AFFECT CD25 AND CD69 EXPRESSION ON CD4⁺ T CELLS

Other proteins investigated included CD25 and CD69, which are both upregulated on the surface of the CD4⁺ T cell upon activation through TCR (Cantrell 1996). BB-3103 treated CD4⁺ T cells upregulated the low affinity IL-2 receptor α chain, CD25, to comparable levels as the no inhibitor controls (Figure 5.4).

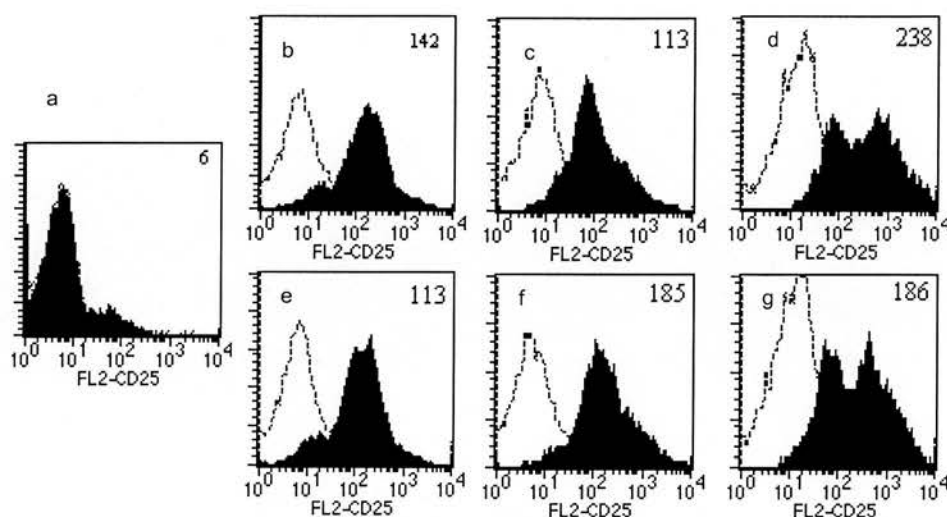


Figure 5. 4: BB-3103 does not affect CD25 expression on WT CD4⁺ T cells. Analysis of CD25 expression on naïve C57BL/6 enriched CD4⁺ T cells activated with immobilised anti-CD3 mAb and soluble anti-CD28 mAb over a period of 3 days in the presence or absence of BB-3103. Panels represent: a) freshly purified CD4⁺ T cells, b) activated CD4⁺ T cells for 24hrs, c) activated CD4⁺ T cells for 48hrs, d) activated CD4⁺ T cells for 72hrs, e) activated CD4⁺ T cells for 24hrs+20 μ M BB-3103, f) activated CD4⁺ T cells for 48hrs+20 μ M BB-3103, g) activated CD4⁺ T cells for 72hrs+20 μ M BB-3103. Dotted lines represent isotype controls, filled profiles represent positively stained cell counts expressing CD25. MFI values for CD25 expression are indicated top right in each panel. The histograms shown are from one experiment and are representative of three animals.

Resting CD4⁺ T cells expressed very low levels of CD25 while it was considerably upregulated upon activation (Figure 5.4). There seemed to be, however, no distinguishable

difference of CD25 expression between BB-3103 treated and control groups. The inhibition of proliferation discussed earlier in this chapter was, therefore, not due to dysregulation of CD25 expression, but possibly due to reduced IL-2 secretion.

Another activation marker, CD69 which is rapidly upregulated on CD4⁺ T cells upon activation was also investigated. The results of CD69 upregulation in the presence or absence of BB-3103 are shown in Figure 5.5 below.

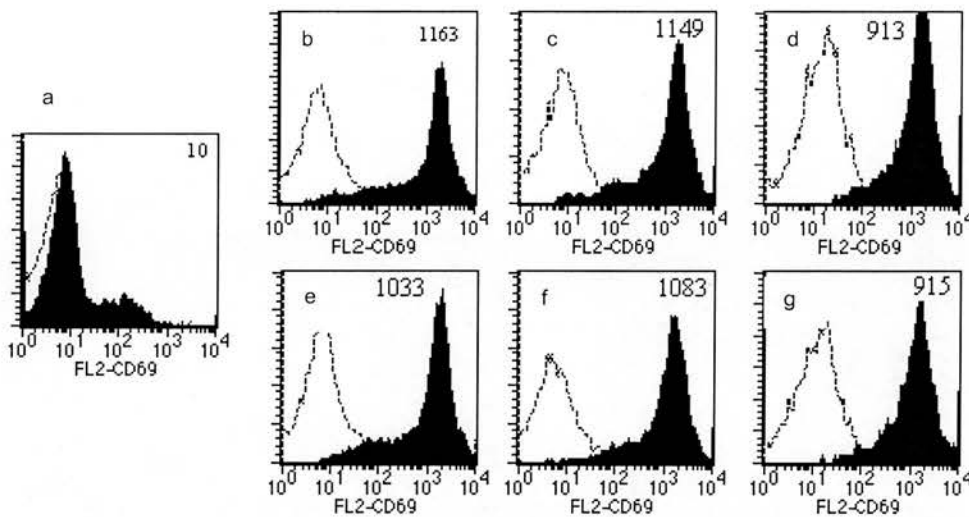


Figure 5. 5: BB-3103 does not affect CD69 expression on WT CD4⁺ T cells. Analysis of CD69 expression on naïve C57BL/6 enriched CD4⁺ T cells activated with immobilised anti-CD3 mAb and soluble anti-CD28 mAb over a period of 3 days in the presence or absence of BB-3103. Panels represent: a) freshly purified CD4⁺ T cells, b) activated CD4⁺ T cells for 24hrs, c) activated CD4⁺ T cells for 48hrs, d) activated CD4⁺ T cells for 72hrs, e) activated CD4⁺ T cells for 24hrs+20 μ M BB-3103, f) activated CD4⁺ T cells for 48hrs+20 μ M BB-3103, g) activated CD4⁺ T cells for 72hrs+20 μ M BB-3103. Dotted lines represent isotype controls, blue-filled profiles represent positively stained cell counts expressing CD69. MFI values for CD69 expression are indicated top right in each panel. The histograms shown are from one experiment and are representative of three animals.

Similarly to CD25 expression, CD69 was also upregulated in activated CD4⁺ T cells in the presence of BB-3103 despite the proliferation being markedly reduced in the presence of the inhibitor (see Chapter 3).

5.2.4 BB-2116 AFFECTS TNF α AND TNFRII RELEASE FROM HUMAN PBMCs *

BB-2116 is another hydroxamate inhibitor which is similar to BB-3103 in that it is broad range and inhibits PWM induced proliferation of human PBMCs to similar extents (GT Layton, personal communication). TNF α and TNFRII are both prime targets for metalloproteinase activity and, therefore, it was investigated whether inhibition of TNF α and TNFRII release was the cause of reduced T cell proliferation. Figure 5.6 below shows results of ELISAs performed for soluble forms of TNF α and TNFRII. Activation mediated release of TNF and TNFRII from PBMCs seemed to be inhibited in the presence of BB-2116.

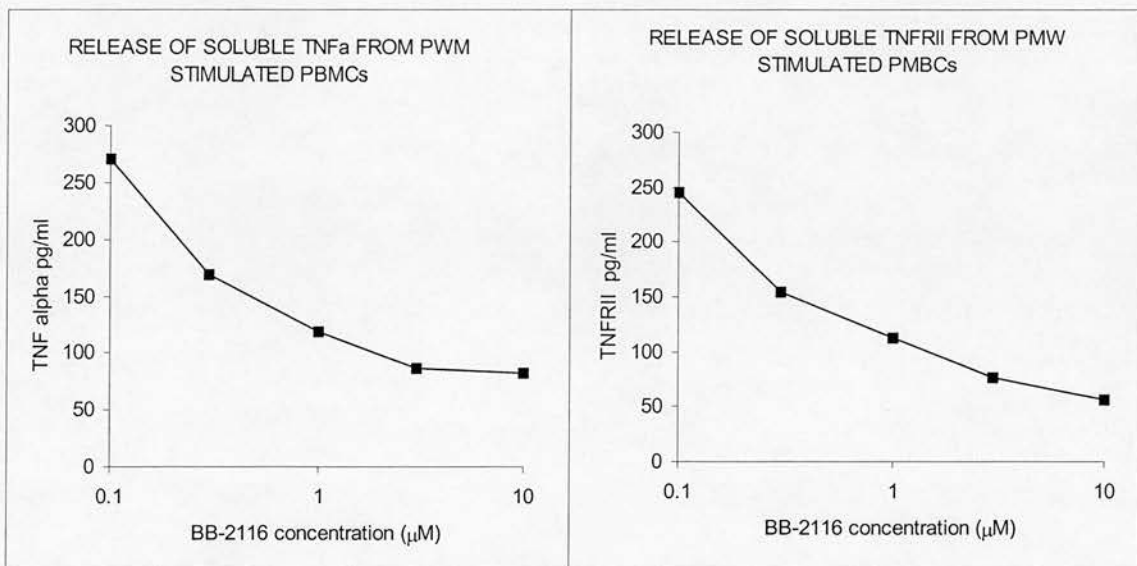


Figure 5. 6: BB-2116 affects TNF α and TNFRII release from human PBMCs. TNF α and TNFRII release was measured from activated human PBMCs (2.5μg/ml PWM for 48hrs) in the presence of BB-2116. BB-2116 inhibited the release of both TNF α and TNFRII upon PBMC activation.

*These experiments were performed by S.J. Harris and I.G Hemingway at British Biotech Plc.

5.2.4.1 EXOGENOUS TNF α DOES NOT RECONSTITUTE PBMC PROLIFERATION*

Since the hydroxamate inhibitor was capable of inhibiting the release of TNF α and TNFRII, the effect of adding soluble TNF α to PWM treated PBMCs in the presence of BB-2116 was investigated. This would determine whether a lack of soluble TNF α within the culture medium could be responsible for downregulating the proliferative response of human T cells to PWM. Human PBMCs were treated with 2.5 μ g/ml PWM and 10 μ M BB-2116 in the presence of 100ng/ml recombinant human TNF α (Figure 5.7).

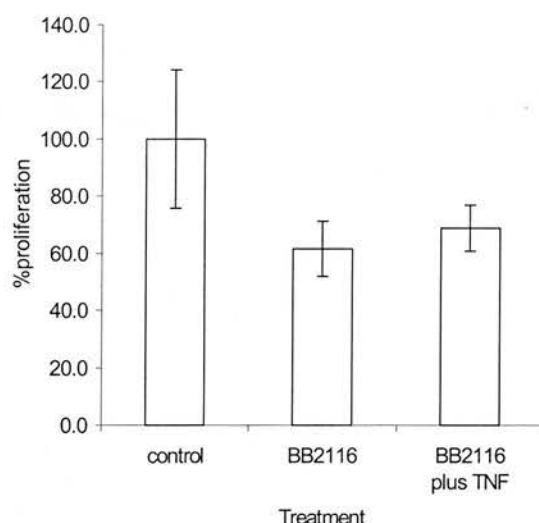


Figure 5. 7: Inhibition of proliferation is not due to inhibition of TNF α release. Proliferation of human PBMCs in the presence of 2.5 μ g/ml PWM (control) and 10 μ M BB-2116 (BB-2116) and 100ng/ml recombinant human TNF α (BB-2116 plus TNF). Addition of BB-2116 inhibited PBMC proliferation as expected and the addition of recombinant TNF α did not restore the proliferation response seen in the absence of BB-2116. No details are known about length of thymidine uptake in this experiment.

The results of these experiments confirm that BB-2116 is capable of inhibiting T cell proliferation to PWM and that addition of recombinant TNF α to the culture did not restore proliferation of PBMCs. These findings hint that the inhibition of soluble TNF α production through metalloproteinase activity may not be responsible for the decrease in proliferation seen to PWM. Soluble TNFRII was not investigated since it has no reported signalling

properties to membrane bound TNF α , but rather acts as a competitor to membrane bound TNFRII.

*These experiments were performed by S.J. Harris and I.G Hemingway at British Biotech Plc.

SUMMARY

- CD25 and CD69 expression was upregulated on both BB-3103 and control samples.

DISCUSSION (CHAPTER 5)

Analysis of cytokine secretion showed that IL-2 production was decreased in the presence of BB-3103, whereas IL-4, IL-5 and IFN γ secretion was unaffected. The observed decrease in IL-2 secretion was the first indication which could clarify the decrease in proliferation of CD4⁺ T cells in the presence of BB-3103 since IL-2 secretion is directly proportional to the amount of proliferation of activated CD4⁺ T cells (Jain *et al.* 1995). This observation would have been extended by addition of exogenous IL-2 to BB-3103 treated T cells, but unfortunately, these experiments were not performed.

Phenotypic analysis revealed that several cell surface molecules were differently regulated in the presence of BB-3103, notably CD27 and CD62L. Both of these proteins were downregulated from the surface of the CD4⁺ T cells in the absence of BB-3103. In the presence of the inhibitor, however, expression of these markers was maintained on the surface suggesting that they may have been shed upon activation, and the enzyme mediating the shedding process was a metalloproteinase. This is the first report that CD27 expression could be regulated by (a) metalloproteinase(s).

CD27 was an appealing candidate to investigate since it is a member of metalloproteinase-sensitive TNFR family and is capable of influencing the activation state of the CD4⁺ T cell. Cross-linking CD27 on the surface of activated CD4⁺ T cells enhances proliferation and increases TNF α release from the cell without affecting IL-2, IL-4 and IL-10 secretion (Hintzen *et al.* 1995; Lens *et al.* 1998; Watts and DeBenedette 1999). Moreover, upon activation, the TNF-family ligand for CD27 (CD70) is transiently expressed on the surface of activated CD4⁺ T cells and thus provides a T-T cell signalling mechanism which enhances proliferation of the CD27 bearing cell (Hintzen *et al.* 1995). Inhibition of CD27 shedding with the use of BB-3103 could therefore be seen to modify the proliferative state of enriched, activated CD4⁺ T cell, although maintaining CD27 on the surface of these cells might have been expected to enhance the proliferative response due to prolonged interactions with CD70. Furthermore, since CD27 signalling does not affect the expression of IL-2 (Watts and DeBenedette 1999), and since IL-2 secretion was reduced in the presence of BB-3103, it seemed unlikely that maintaining CD27 on the cell surface could be responsible for inhibiting CD4⁺ T cell proliferation. To verify that CD27 was actually shed from the surface of the lymphocytes, an ELISA assay should have been performed to ensure that soluble CD27 was present in the culture medium and that this concentration of sCD27 was downregulated in the presence of BB-3103.

CD62L was also inhibited from being downregulated upon activation in the presence of BB-3103, this observation had already been reported with other inhibitors of metalloproteinases (Feehan *et al.* 1996; Preece *et al.* 1996; Walcheck *et al.* 1996). Upon activation, CD4⁺ T cells shed CD62L which was inhibited in the presence of BB-3103. CD62L has been implicated in signalling events in neutrophils upon ligation with natural or synthetic ligands (Palecanda *et al.* 1992) and thus capable of increasing cytosolic calcium flux and activate MAPK signalling cascade (Brenner *et al.* 1996; Laudanna *et al.* 1994; Waddell *et al.* 1995; Brenner *et al.* 1997). CD62L has also been found associated with p56^{lck}, CD3 complex and

calmodulin (Brenner *et al.* 1996; Murakawa *et al.* 1992) and inhibition of shedding of CD62L could potentially modify the activation state of these molecules although this has never been demonstrated to date. Similarly to CD27 generation of a soluble form of CD62L should have been investigated by performing ELISAs with the culture supernatant. If CD62L was indeed shed, we would expect the soluble form of CD62L to be reduced in the presence of BB-3103.

Activation of CD4⁺ T cells in the presence of BB-3103 significantly decreased proliferation but had no effect on cell surface markers which are upregulated upon activation of T lymphocytes such as CD25 (IL-2 receptor α chain) and CD69 (activation inducer molecule (AIM)). CD25 expression has been demonstrated to be maintained on the surface of T lymphocytes by activated STAT5 (A and B) although initial expression of CD25 is regulated by TCR activation (Nakajima *et al.* 1997). Maintenance of CD25 expression on T lymphocytes thus works in a cyclic pattern whereby ligation of IL-2 to IL2R initiates STAT5 activation which in turn induces expression of IL2R. Taken together these data suggest that presence of BB-3103 in proliferation assays cannot affect STAT5 A/B activation. The starting population of cells which was used in all these experiments demonstrated a small percentage of cells expressing CD25 and CD69. This could be attributed to either, infections in the animals used, thus generating a small population of activated T cells, or activation of cells by the method used for preparation of enriched CD4⁺ T cell populations which could non-specifically activate CD4⁺ T cells.

Expression of CD69 is regulated by activated Ras (D'Ambrosio *et al.* 1994). Ras plays an integral role in TCR mediated signalling (Cantrell 1996; Hallberg *et al.* 1994) and Figure 1.11) and upon activation, induces CD69 expression. In the presence of BB-3103, however, CD69 expression was not downregulated which therefore suggests that Ras activation may not directly be modified in the presence of BB-3103.

Metalloproteinase mediated shedding of TNF α and TNFRII have also been reported (Black *et al.* 1997) and shown in this thesis to be inhibited by BB-2116. Since TNFRII mediated signalling is involved in anti-apoptotic pathways ((Rothe *et al.* 1995) and Figure 1.6), it would have been expected that maintaining the receptor on the surface of lymphocytes upon activation in the presence of inhibitor, would enhance lymphocyte survival and therefore either increase proliferation counts or have no effect. This was not the case, however, suggesting that inhibition of TNFRII shedding was not responsible for downregulating proliferation. The effect of inhibiting TNF α shedding was also investigated by reconstituting proliferation assays with recombinant soluble TNF α , but, showed no variance between inhibitor with or without exogenous TNF α . These data loosely indicated that inhibition of TNF α shedding may not have been the causative factor in inhibition of proliferation in the presence of BB-3103. Unfortunately, modulation of TNFR1 shedding in the presence of inhibitor was not investigated but since signalling through TNFR1 induces apoptotic events (Figure 1.6 and (Wallach *et al.* 1998) but since no increase in apoptosis was observed by AnnexinV binding, it is unlikely that modulation of TNF α /TNFR1 signalling was responsible for inhibition of CD4⁺ T cell proliferation. These experiments, performed at British Biotech, are, however, incomplete and do not answer the question addressed by this thesis. Firstly, BB-2116 is a different inhibitor to BB-3103 and although they are both broad range hydroxamate inhibitors, they cannot be compared. The other issue involves the use of species. These experiments were performed on human peripheral blood lymphocytes and therefore do not necessarily correlate to the immune response which may be observed in mouse cells. To address these issues, similar experiments should be performed on PWM activated mouse splenocytes with BB-3103 and to confirm inhibition of shedding, flow cytometry should be performed on the cells to determine whether TNF α and TNFRII expression on the splenocytes is maintained. The results shown in Figure 5.7, although suggesting that inhibition of TNF α shedding may not play a role in cell proliferation in the presence of a hydroxamate inhibitor, should have included controls showing cells activated

by PWM in the presence of TNF α as well as cells in the presence of TNF α alone, to determine their effect in the absence of inhibitor. No data was provided showing background proliferation of cells in the absence of PWM or TNF α and therefore we cannot determine whether the proliferation caused was not due to contamination or another problem. In these experiments, 100ng/ml of recombinant TNF α was used which is a very high concentration in terms of TNF related molecules. Further experiments showing a dose response curve to TNF α and TNF α in the presence of BB-3103 would have been more informative.

The results shown in Fig 5.7 are presented as percentage of inhibition which does not provide enough information as to the state of the cells in the assay. Presenting the data in cpm of tritiated thymidine incorporation would have been more appropriate.

CHAPTER 6

6.1 PROLIFERATION OF CD4⁺ T CELLS FROM CD62L GENE DEFICIENT ANIMALS IS NOT INHIBITED BY BB-3103

The effect of BB-3103 was investigated on the proliferation of enriched CD4⁺ T cells isolated from CD62L gene deficient (C57BL/6) mice. This was performed determine whether the inhibition of CD62L shedding was responsible of downregulating CD4⁺ T cell proliferation. CD4⁺ T cells were isolated from naïve (unimmunised) wild type (WT) spleens and naïve CD62L gene deficient (CD62L KO) spleens. The purified cells were activated using immobilised anti-CD3 mAb and 5µg/ml soluble anti-CD28 mAb, over a period of 3 days as previously described for WT CD4⁺ T cells. The proliferative response obtained from WT and CD62L deficient animals in the presence of BB-3103 is shown in Figure 6.1.

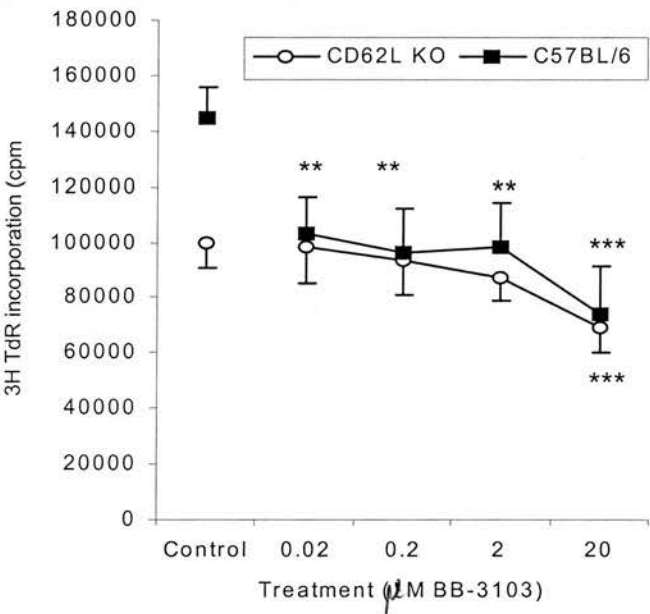


Figure 6. 1: CD4⁺ T cell proliferation from WT and CD62L KO animals in the presence of BB-3103. Graph representing immobilised anti-CD3 mAb and soluble anti-CD28 mAb induced proliferation of purified CD4⁺ T cells from WT or CD62L deficient animals in the presence of BB-3103. Proliferation was assessed by thymidine incorporation for the last 16hrs of culture. The WT CD4⁺ T cell proliferation was inhibited by BB-3103 (■) as previously observed, whereas the CD62L gene deficient CD4⁺ T cells were not affected by BB-3103 (○). Graph represents mean of four animals. Background proliferation did not exceed 5000cpm. p values compare BB-3103 treated

samples to respective control groups.

The CD62L deficient CD4⁺ T cells failed to proliferate to the same extent as the WT CD4⁺ T cells in the control samples. It also appeared that the CD62L deficient CD4⁺ T cells were not affected by BB-3103. Furthermore, proliferation of WT CD4⁺ T cells in the presence of BB-3103 was reduced to that of the CD62L deficient groups. This latter finding is highly suggestive that CD62L on CD4⁺ T cells plays a critical role in promoting proliferation.

SUMMARY

- T cell proliferation in CD62L deficient animals was
 - downregulated compared to wild type CD4⁺ T cells
 - not inhibited by BB-3103

6.2 CYTOKINE SECRETION FROM CD62L KO CD4⁺ T CELLS IS NOT AFFECTED BY BB-3103

Cytokine secretion by purified, activated CD4⁺ T cells isolated from CD62L deficient animals was measured at 48hrs and showed no variation in the presence of BB-3103 (Figure 6.2).

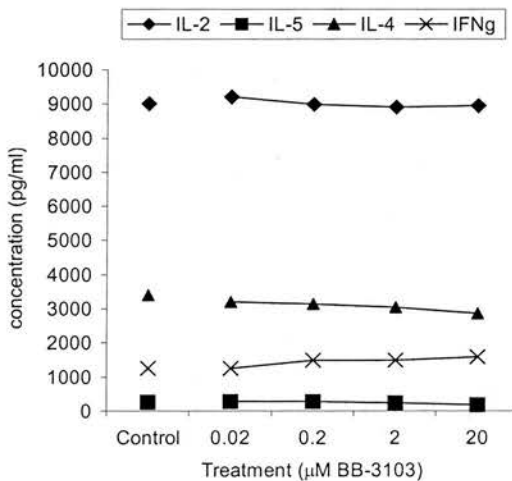


Figure 6. 2: Cytokine secretion of activated CD62L KO CD4⁺ T cells in the presence of BB-3103: Graph representing IL-2, IL-5, IL-4 and IFN γ concentrations in supernatants of activated CD62L KO CD4⁺ T cells as detected by ELISA. All cytokines tested showed no variation in the presence of inhibitor. Graph represents mean of two animals. IL-5 concentrations fell beneath detection level and may not represent actual IL-5 concentrations in culture supernatants.

6.3 PHENOTYPIC ANALYSIS OF CD62L DEFICIENT CD4⁺ T CELLS

CD62L deficient CD4⁺ T cells failed to proliferate to the same extent as WT cells regardless of the presence of BB-3103, therefore, the expression of activation antigens on the CD62L deficient CD4⁺ T cells was analysed. The histogram in Figure 6.3a verifies that CD62L is absent from the surface of the cell as described previously (Arbonés *et al.* 1994). MFI values do not necessarily represent a poisson distribution staining pattern as can be seen in Fig 6.3b, where 2 peaks are evident.

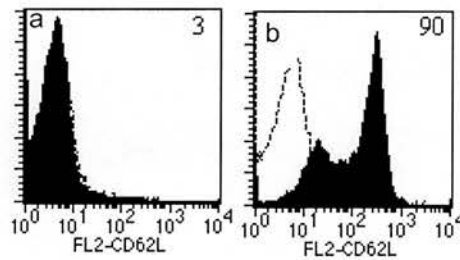


Figure 6. 3: CD62L expression on CD62L gene deficient CD4⁺ T cells. (a)Analysis of CD62L expression on purified CD62L deficient CD4⁺ T cells as compared to CD62L expression on wild type CD4⁺ T cells (b).. The MFI value for CD62L staining is shown in the top right hand corner of the panel and is comparable to the isotype control staining pattern (MFI isotype=3.91)

6.3.1 PHENOTYPIC ANALYSIS OF CD62L KO CD4⁺ T CELLS IN THE PRESENCE OF BB-3103

Analysis of cell surface proteins expressed on activated CD62L KO CD4⁺ T cells in the presence or absence of BB-3103 are summarised below in Table 6.1. As in Table 5.1, use of MFI values is not representative of poisson distribution, but represents total fluorescence of cell populations which may contain 2 or more fluorescence intensities (see Fig. 6.4 for example). The use of MFI in this thesis is used as a mean of interpreting general effects by BB-3103 rather than describing a poisson distribution of staining.

	day 0	day 1		day 2		day 3	
	ko	ko		ko		ko	
		control	20 μ M BB	control	20 μ M BB	control	20 μ M BB
CD25	5.38	104.13	106.19	123.68	109.62	139.6	141.24
CD69	7.81	785.8	883.1	1202.35	1180	1005.46	884.52
CD40L	6.24	9.8	16.1	13.79	18.96	23.4	32.01
Fas	84.75	123.53	121.6	236.2	218.72	379.36	328.49
FasL	5.17	10.31	9.88	10.15	10.04	12.24	16.09
CD62L	3.71	9.1	10.41	9.52	10.18	12.37	11.98
CD27	362.24	181.88	393.42	312.17	539.52	330.39	608.3
CD30	11.68	11.17	20.89	30.39	28.59	69.63	54.95
4-1BB	9.42	34.05	29.5	43.56	50.46	70.9	69.88

Table 6. 1: Summary of the phenotypic data acquired by flow cytometry on purified CD62L KO CD4⁺ T cells activated with immobilised anti-CD3 mAb and 5 μ g/ml soluble anti-CD28 mAb in the presence or absence of 20 μ M BB-3103. The numbers indicate MFI values found for each marker. Results show that CD27 expression is highly upregulated in the presence of BB-3103. Shaded markers are shown in more detail later in the text. Values indicated in the table are representative of triplicates.

6.3.1 BB-3103 DOES NOT MODULATE CD25 EXPRESSION ON CD62L KO CD4⁺ T CELLS

The expression pattern of CD25 on CD62L deficient CD4⁺ T cells in the presence of BB-3103 is presented in Figure 6.4.

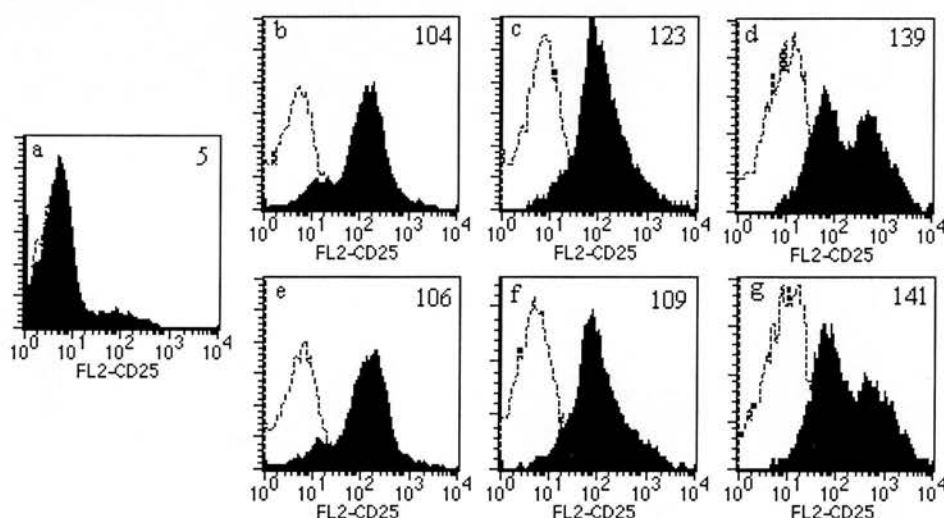


Figure 6. 4: BB-3103 does not affect CD25 expression on CD62L KO CD4⁺ T cells. Analysis of CD25 expression on naïve CD62L deficient enriched CD4⁺ T cells activated with immobilised anti-CD3 mAb and soluble anti-CD28 mAb over a period of 3 days in the presence or absence of BB-3103. Panels represent: a) freshly purified CD4⁺ T cells, b) activated CD4⁺ T cells for 24hrs, c) activated CD4⁺ T cells for 48hrs, d) activated CD4⁺ T cells for 72hrs, e) activated CD4⁺ T cells for 24hrs+20 μ M BB-3103, f) activated CD4⁺ T cells for 48hrs+20 μ M BB-3103, g) activated CD4⁺ T cells for 72hrs+20 μ M BB-3103. Dotted lines represent isotype controls, filled profiles represent positively stained cell counts expressing CD25. MFI values for CD25 expression are indicated top right in each panel. The histograms shown are from one experiment and are representative of 3 different animals.

Expression of CD25 on CD62L deficient CD4⁺ T cells was not modified in the presence of BB-3103 (Figure 6.4). It would therefore appear that the reduced proliferation displayed by CD62L deficient CD4⁺ T cells in the absence of BB-3103 is not due to diminished expression of CD25 upon activation.

6.3.2 BB-3103 DOES NOT REDUCE CD69 EXPRESSION ON CD62L KO CD4⁺ T CELLS

CD69 expression was also investigated on the surface of activated CD62L deficient CD4⁺ T cells and was upregulated regardless of the presence of BB-3103 (Figure 6.5).

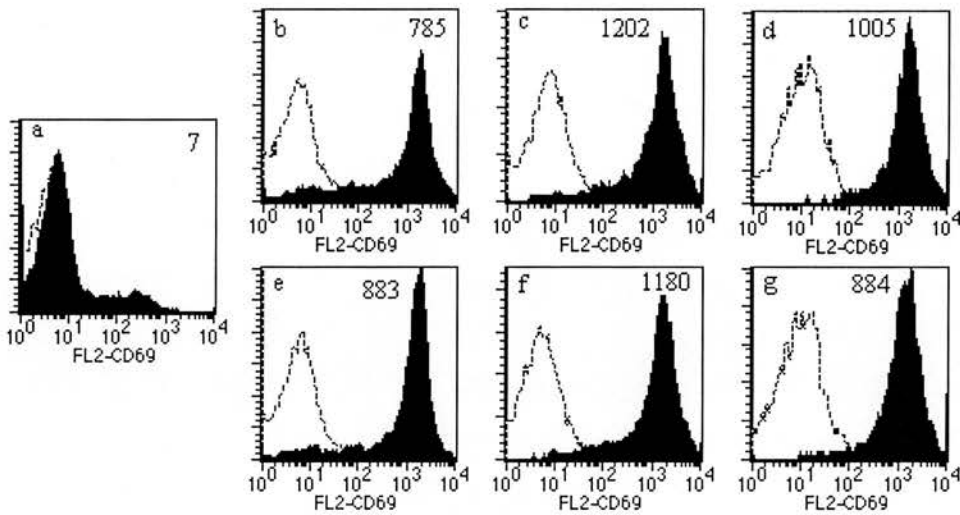


Figure 6. 5: BB-3103 does not affect CD69 expression on CD62L deficient CD4⁺ T cells. Analysis of CD69 expression on naïve CD62L deficient enriched CD4⁺ T cells activated with immobilised anti-CD3 mAb and soluble anti-CD28 mAb over a period of 3 days in the presence or absence of BB-3103. Panels represent: a) freshly purified CD4⁺ T cells, b)activated CD4⁺ T cells for 24hrs, c)activated CD4⁺ T cells for 48hrs, d)activated CD4⁺ T cells for 72hrs, e)activated CD4⁺ T cells for 24hrs+20μM BB-3103, f)activated CD4⁺ T cells for 48hrs+20μM BB-3103, g)activated CD4⁺ T cells for 72hrs+20μM BB-3103. Dotted lines represent isotype controls, filled profiles represent positively stained cell counts expressing CD69. MFI values for CD69 expression are indicated top right in each panel. The histograms shown are from one experiment and are representative of 3 different animals.

Despite the reduced proliferation of CD62L deficient CD4⁺ T cells, both activation markers tested (CD25 and CD69) were upregulated indicating that the CD4⁺ T cells were being activated.

SUMMARY

- CD25 and CD69 expression on CD62L KO CD4⁺ T cells was comparable to WT cells and was not modulated by BB-3103.

6.3.3 BB-3103 AFFECTS CD27 EXPRESSION ON CD62L KO CD4⁺ T CELLS

Since the expression of CD27 was modified in the presence of BB-3103 in WT CD4⁺ T cells, the analysis was extended to CD4⁺ T cells from CD62L deficient mice in the presence of BB-3103. The expression pattern observed was similar to that seen in WT CD4⁺ T cells in the presence of BB-3103 (Figure 6.6).

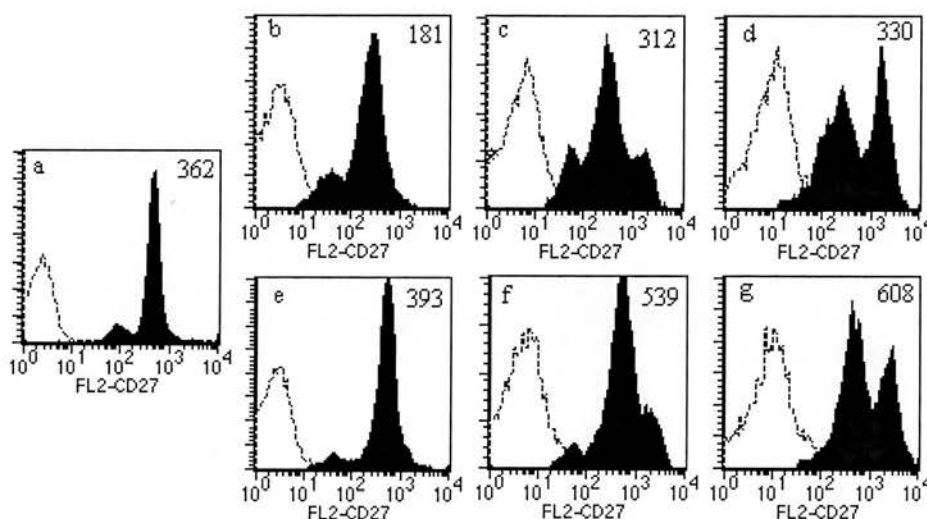


Figure 6. 6: BB-3103 affects CD27 expression on CD62L deficient CD4⁺ T cells. Analysis of CD27 expression on naïve CD62L deficient enriched CD4⁺ T cells activated with immobilised anti-CD3 mAb and soluble anti-CD28 mAb over a period of 3 days in the presence or absence of BB-3103. Panels represent: a) freshly purified CD4⁺ T cells, b) activated CD4⁺ T cells for 24hrs, c) activated CD4⁺ T cells for 48hrs, d) activated CD4⁺ T cells for 72hrs, e) activated CD4⁺ T cells for 24hrs+20 μ M BB-3103, f) activated CD4⁺ T cells for 48hrs+20 μ M BB-3103, g) activated CD4⁺ T cells for 72hrs+20 μ M BB-3103. Dotted lines represent isotype controls, filled profiles represent positively stained cell counts expressing CD27. MFI values for CD27 expression are indicated top right in each panel. The histograms shown are from one experiment and are representative of 3 different animals.

CD27 expression was high on CD62L deficient CD4⁺ T cells and upon activation, was downregulated by metalloproteinase activity, since on the BB-3103 treated samples, the

expression of CD27 was maintained at high levels, and showed similar patterns of expression as on the WT CD4⁺ T cells (compare Figure 6.6 with Figure 5.2).

6.4 SOLUBLE ANTI-CD27 mAb INCREASES PROLIFERATION IN WT AND CD62L DEFICIENT CD4⁺ T CELLS

Although the evidence so far strongly suggested CD62L on CD4⁺ T cells was a primary target for BB-3103 and that this led to reduced proliferation, the effect of activating the CD27 pathway in conjunction with TCR mediated signalling was investigated. Proliferation of WT CD4⁺ T cells purified from a naïve spleen was increased in the presence of various doses of agonist anti-CD27 mAb (Figure 6.7).

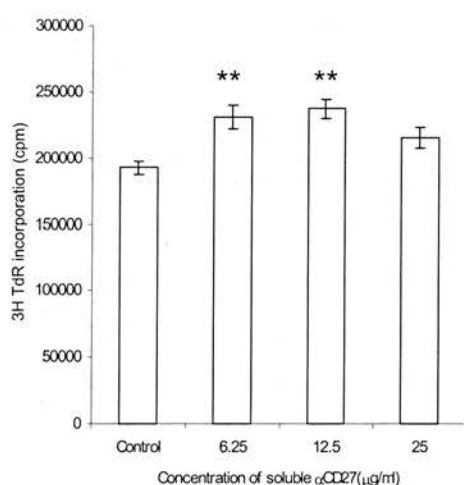


Figure 6. 7: Soluble anti-CD27 mAb increases proliferation of WT CD4⁺ T cells. Purified wild type CD4⁺ T cells stimulated with immobilised anti-CD3 mAb and 5 μ g/ml soluble anti-CD28 mAb in the presence of varying concentrations of soluble agonist anti-CD27 mAb. Results indicated an increase in proliferation when CD27 is cross-linked on the T cell surface (~30% increase). Interestingly, higher doses of soluble anti-CD27 mAb (25 μ g/ml) were not as effective in increasing the proliferative state of the T cells as compared to lower doses (12.5 and 6.35 μ g/ml). Graph represents mean of three animals. Proliferation was assessed by thymidine incorporation for the last 16hrs of culture. Background proliferation did not exceed 5000cpm.

As previously reported (Gravestien *et al.* 1995; van Lier *et al.* 1987) activation of the CD27 pathway in conjunction with TCR mediated signalling enhances proliferation in CD4⁺ T cells. Similar results were obtained from binding CD27 on the surface of CD62L deficient CD4⁺ T cells (Figure 6.8).

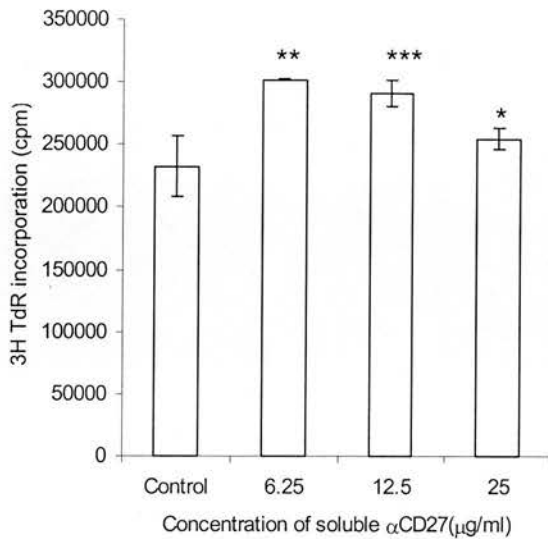


Figure 6. 8: Soluble anti-CD27 mAb increases proliferation of CD62L deficient CD4⁺ T cells. Purified CD62L deficient CD4⁺ T cells activated with immobilised anti-CD3 mAb and 5 μ g/ml soluble anti-CD28 mAb in the presence of varying concentrations of soluble agonist anti-CD27 mAb. Results indicated an increase in proliferation of CD62L deficient CD4⁺ T cells when CD27 was cross-linked on the T cell surface (~17% increase). Similarly to WT CD4⁺ T cells, higher doses of anti-CD27 mAb (25 μ g/ml) were not as effective in increasing the proliferative state of the T cells as compared to lower doses (12.5 and 6.35 μ g/ml). Proliferation was assessed by thymidine incorporation for the last 16hrs of culture. Graph represents mean of three animals. Background proliferation

did not exceed 5000 cpm.

SUMMARY

- Cross-linking CD27 with monoclonal antibodies increases proliferation of WT and CD62L KO CD4⁺ T cells

6.5 BB-3103 INHIBITS ANTI-CD27 MEDIATED PROLIFERATION INCREASE IN WT CD4⁺ T CELLS

Since addition of soluble agonist anti-CD27 mAb to cultures increased both WT and CD62L deficient CD4⁺ T cell proliferation, the effect of inhibition of CD27 shedding by addition of BB-3103 to similar proliferation conditions was determined.

Proliferation of purified WT CD4⁺ T cells with 6.25µg/ml soluble agonist anti-CD27 mAb in conjunction with a BB-3103 titration was measured. This concentration of anti-CD27 mAb was selected as this was the dose which caused WT CD4⁺ T cells to proliferate maximally (see Figure 6.9).

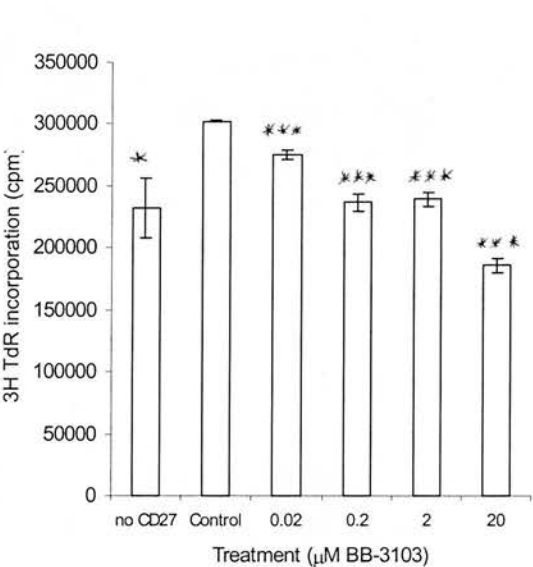


Figure 6. 9: Proliferation of WT CD4⁺ T cells in the presence of soluble anti-CD27 mAb and BB-3103. Graph showing that addition of BB-3103 to proliferation assays in the presence of 6.25µg/ml anti-CD27 mAb was still capable of decreasing wild type CD4⁺ T cell proliferation. 'No CD27' bar indicates WT CD4⁺ T cell proliferation using immobilised anti-CD3 mAb and 5µg/ml soluble anti-CD28 mAb only. 'Control' bar represents WT CD4⁺ T cells activated with anti-CD3 mAb and anti-CD28 mAb in conjunction with 6.25µg/ml anti-CD27 mAb. The molarity values indicate the concentration of BB-3103 in samples containing immobilised anti-CD3 mAb and soluble anti-CD28 mAb and soluble anti-CD27 mAb. p values shown are relevant only to the 'Control' results which contain

6.25µg/ml soluble anti-CD27 mAb and no BB-3103. Proliferation was assessed by thymidine incorporation for the last 16hrs of culture. Graph represents mean of three animals. Background proliferation did not exceed 10000 cpm.

Results demonstrate that the increase in proliferation of WT CD4⁺ T cells is due to the presence of anti-CD27 mAb. Titration of BB-3103 into these assays, however, demonstrated that although the CD27 pathway is activated, the inhibitor was still capable of decreasing proliferation.

6.6 BB-3103 DOES NOT INHIBIT ANTI-CD27 mAb MEDIATED PROLIFERATION INCREASE IN CD62L KO CD4⁺ T CELLS

A similar experiment was applied to CD62L deficient CD4⁺ T cells and revealed that titration of BB-3103 into these experiments had no effect on these CD4⁺ T cells (Figure 6.10).

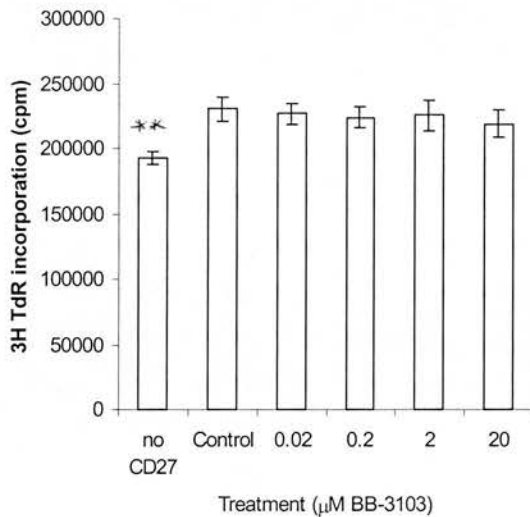


Figure 6. 10: Proliferation of CD62L KO CD4⁺ T cells in the presence of soluble anti-CD27 mAb and BB-3103. Graph showing that addition of BB-3103 to proliferation assays (anti-CD3/anti-CD28 mAbs) in the presence of 6.25μg/ml soluble anti-CD27 mAb was incapable of decreasing CD4⁺ T cell proliferation isolated from CD62L deficient animals. 'No CD27' bar indicates CD62L deficient CD4⁺ T cell proliferation using immobilised anti-CD3 mAb and soluble anti-CD28 mAbs only. 'Control' bar represents CD62L deficient CD4⁺ T cells activated with anti-CD3 mAb and anti-CD28 mAbs in conjunction with 6.25μg/ml anti-CD27 mAb. The molarity values indicate the concentration of BB-3103 in samples

containing anti-CD3/anti-CD28 mAbs and anti-CD27 mAb. p values shown are relevant only to the control results which contain anti-CD27 mAb and no BB-3103. Proliferation was assessed by thymidine incorporation for the last 16hrs of culture. Graph represents mean of three animals. Background proliferation did not exceed 10000cpm.

Soluble agonist anti-CD27 mAb increased the proliferation of CD62L deficient CD4⁺ T cells (Figure 6.8). However, addition of BB-3103 did not reduce proliferation as seen in the WT CD4⁺ T cells (Figure 6.9). This finding again strongly suggests that maintaining CD27 on the surface of CD4⁺ T cells upon activation or addition of an agonist mAb, and BB-3103 plays no role in reducing the subsequent proliferation of the cells.

SUMMARY

- BB-3103 reduced WT CD4⁺ T cell proliferation even in the presence of anti CD27 mAb, but had no effect on CD62L deficient CD4⁺ T cells

DISCUSSION (CHAPTER 6)

When added to proliferation assays with cells purified from CD62L deficient animals, BB-3103 did not reduce proliferation. This was evidence that presence of CD62L on the surface of CD4⁺ T cells can regulate the amount of proliferation and that inhibition of CD62L shedding (in the presence of the inhibitor with BB-3103), reduces proliferation to similar levels observed in CD62L KO. Evidence that CD62L KO CD4⁺ T cells do not proliferate to the same extent in the absence of BB-3103 suggest that the association of CD62L with CD3complex and p56^{lck} (Brenner *et al.* 1996; Murakawa *et al.* 1992) is an important determinant in regulating the amount of activation and subsequent proliferation.

Investigation of cytokine secretion from CD62L deficient CD4⁺ T cells in the presence or absence of BB-3103 showed no variation in IL-4, IL-5 and IFN γ secretion as determined in the wild type CD4⁺ T cells. IL-2 secretion which was affected in the presence of BB-3103 in wild type CD4⁺ T cells was not affected in CD62L deficient CD4⁺ T cells despite the presence of BB-3103. The inability of BB-3103 to modulate IL-2 secretion in the CD62L deficient cells could correspond to the proliferative response since the proliferation of CD62L KO CD4⁺ T cells were not affected by BB-3103.

The values shown in Table 6.1, are, like Table 5.1, not representative of a poisson distribution of staining, and in some instances shows the mean fluorescence of two or more distinct populations expressing various amounts of protein. Therefore, the MFI values

should not be interpreted as anything else but indicative of a trend rather than a staining pattern.

Similarly to wild type CD4⁺ T cells, the expression of activation markers such as CD25 and CD69 were not modulated in the presence of BB-3103, again, suggesting that the JAK3/STAT5 A/B pathway was not affected (for expression and maintenance of CD25 at the cell surface (Nakajima *et al.* 1997)) and that normal activation of Ras was occurring for the expression of CD69 (D'Ambrosio *et al.* 1994). Similarly to CD62L⁺ cells, the starting population always demonstrated a small percentage of CD25 expressing cells. This population cannot be attributed to anything else than an infection in the animals used for these experiments or a non-specific activation process occurring during the enrichment of lymphocytes prior to activation.

Expression of CD27 on the CD62L deficient cells was modified in a similar fashion to that observed in the wild type CD4⁺ T cells in the presence of BB-3103 (CD27 shedding was inhibited) despite the observation that CD62L deficient cells were not affected by BB-3103.

Cross-linking CD27 on the surface of the T cells in conjunction with TCR mediated activation (wild type and CD62L KO CD4⁺ T cells) increased the proliferation as described before (Lens *et al.* 1998; Watts, DeBenedette 1999; Hintzen *et al.* 1995) but in the presence of BB-3103 wild type CD4⁺ T cell proliferation was still inhibited whilst CD62L deficient cells remained unaffected. These data were further evidence of the non-involvement of CD27 signalling pathway in proliferation reduction in the presence of BB-3103.

Ligating CD27 with monoclonal antibodies may have had the same effect as ligating with CD70 which is also transiently expressed on activated T cells (Hintzen *et al.* 1995). Wild type CD4⁺ T cells were still inhibited in the presence of BB-3103 when the CD27 pathway

was engaged which suggests that modulation of the interaction of CD27 with CD70 was not the causative factor in reducing T cell proliferation.

The following experiments were designed to further investigate the role of CD62L on T lymphocytes.

CHAPTER 7

7.1 AGONIST ANTI-CD62L mAb INCREASES PROLIFERATION IN WT CD4⁺ T CELLS

Since CD62L deficient CD4⁺ T cells were not modulated by BB-3103, the role of CD62L on WT CD4⁺ T cells was examined. This question was addressed by activating WT CD4⁺ T cells using immobilised anti-CD3 mAb and 5µg/ml soluble anti-CD28 mAb in the presence of varying concentration of soluble anti-CD62L mAb (Figure 7.1).

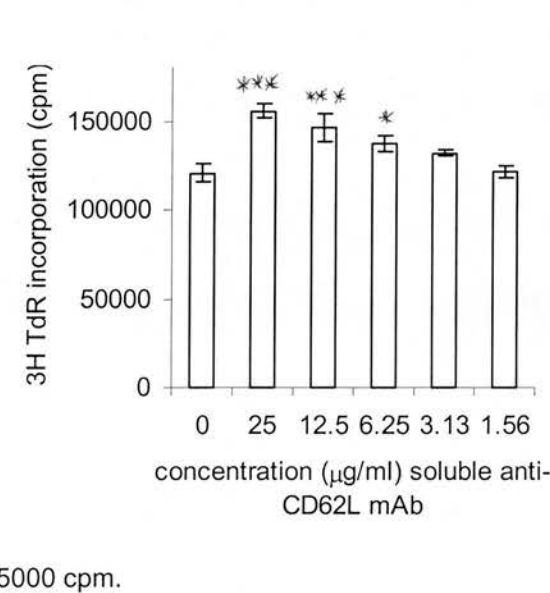


Figure 7. 1: Proliferation of WT CD4⁺ T cells in the presence of plate bound anti-CD3 mAb and soluble anti-CD28 mAb with soluble anti-CD62L mAb. Graphical representation of proliferating CD4⁺ T cells with immobilised anti-CD3 mAb and 5µg/ml soluble anti-CD28 mAb in the presence of various doses of soluble anti-CD62L mAb. A linear correlation between concentration of soluble anti-CD62L mAb and increase in proliferation was observed, with the highest three doses used (25, 12.5 and 6.25µg/ml) being statistically relevant. Proliferation was assayed on day 3 by thymidine incorporation for the last 16hrs of culture. Graph represents means of three animals. Background proliferation did not exceed

The results demonstrate that adding agonist anti-CD62L mAb to WT CD4⁺ T cells in conjunction with TCR mediated signalling increases the proliferation of the CD4⁺ T cells, supporting data obtained by other groups (Murakawa *et al.* 1992).

SUMMARY

- Addition of soluble anti-CD62L monoclonal antibody increased proliferation of CD4⁺ T cells by 30% at highest concentration used of 25µg/ml.

7.2 BB-3103 HAS NO EFFECT ON WT CD4⁺ T CELL PROLIFERATION WHEN CD62L IS PROVIDED WITH A LIGAND

The next experiments were designed to assess the effect of cross-linking CD62L on the surface of the CD4⁺ T cell in conjunction with TCR mediated signalling whilst inhibiting shedding of CD62L with the presence of BB-3103. The addition of BB-3103 to T cells which were activated through TCR in the presence of soluble 12.5 µg/ml anti-CD62L mAb, maintained the increase in proliferation (Figure 7.2).

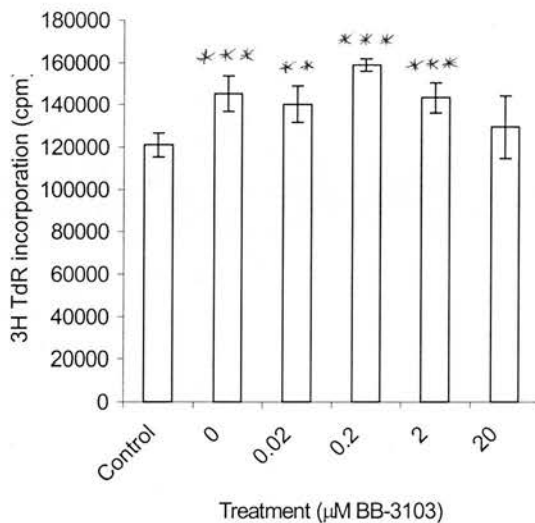


Figure 7. 2: BB-3103 has no effect on WT CD4⁺ T cell proliferation when CD62L is provided with a ligand. Proliferation of CD4⁺ T cells in the presence of immobilised anti-CD3 mAb and 5µg/ml soluble anti-CD28 mAb with 12.5µg/ml soluble anti-CD62L mAb. Proliferation of CD4⁺ T cells in the absence of anti-CD62L mAb is the control bar. The increase in proliferation seen upon addition of anti-CD62L mAb was maintained in the presence of BB-3103 despite the inhibition of shedding. Proliferation assayed on day 3 by thymidine incorporation. Graph represents mean of four animals. Background proliferation did not exceed 5000 cpm.

These results demonstrate that it was possible to protect the proliferation of WT CD4⁺ T cells from the inhibitory effect of BB-3103 if CD62L was provided with a ligand.

SUMMARY

- BB-3103 has no inhibitory effect on CD4⁺ T cell proliferation if CD62L is provided with a ligand (in this case anti-CD62L mAb).

DISCUSSION (CHAPTER 7)

As previously described in other studies, ligation of CD62L on T-lymphocytes with the use of monoclonal antibodies increased proliferation of T cells in a dose dependent manner (Murakawa *et al.* 1992). Similarly, investigation of the effect of cross-linking CD62L in the absence of TCR mediated activation revealed that CD62L was shed (Kahn *et al.* 1998) and that this process was inhibited by BB-3103.

Two processes of CD62L shedding were apparent, TCR mediated shedding and CD62L cross-linking shedding which were both inhibited by BB-3103 suggesting that the enzymes responsible for both events were one or more metalloproteinase.

To investigate the role of CD62L cross-linking further, BB-3103 was added to proliferation assays which contained soluble anti-CD62L antibodies. Unexpectedly, it emerged that cross-linking CD62L on the surface of the T lymphocytes and inhibiting the expected shedding event by addition of BB-3103, protected CD4⁺ T cell proliferation from the inhibitory effects of BB-3103.

Although the latter results are intriguing, they do not explain the observations made in the proliferation assays shown earlier in the chapter 3 since CD62L was never ligated. Ligands for CD62L are expressed by endothelial cells and high endothelial venules. The ligands include Glycam-1 Madcam-1, CD34 and various other highly glycosylated molecules (T. Tedder web site <http://www.ncbi.nlm.nih.gov/PROW/cd/cd62l.htm>).

The data presented above demonstrate that cross-linking CD62L also plays an important role in augmenting signal transduction to the lymphocyte, and, once cross-linked, inhibition of

ligand induced CD62L shedding cannot modify the outcome of the increased signal transduction.

CHAPTER 8

8.1 THE EFFECT OF BB-3103 ON TYROSINE PHOSPHORYLATION OF SIGNALLING MOLECULES

8.1.1 p56^{Lck} PHOSPHORYLATION IS NOT AFFECTED BY BB-3103

Since BB-3103 inhibited CD4⁺ T cell proliferation through mechanisms other than cytotoxicity or apoptosis, BB-3103 was investigated for its ability to alter TCR mediated signalling pathways induced by ligation with anti-CD3 ϵ monoclonal antibodies which would eventually lead to IL-2 production.

A primary event upon TCR stimulation is activation (dephosphorylation) of p56^{Lck} Src tyrosine kinase (Cartwright *et al.* 1987; Kmiecik and Shalloway 1987). The following experiments were designed to investigate the phosphorylation state of p56^{Lck} upon anti-CD3 ϵ monoclonal antibody stimulation in the presence or absence of BB-3103 on WT and CD62L deficient CD4⁺ T cells. Purified CD4⁺ T cells were plated onto immobilised anti CD3 ϵ (20 μ g/ml) for one minute (5x10⁶ cells/sample). Cells were lysed in Tris based NP-40 lysis buffer. Lysates were immunoprecipitated with murine monoclonal anti-phosphotyrosine antibody, PY-99. Immunoprecipitations were loaded onto 8% SDS-PAGE gel, transferred to nitrocellulose and probed with rabbit anti-p56^{Lck} mAb. Detection antibody was goat anti-rabbit HRP conjugated mAb. The p56^{Lck} immuno-blot and densitometry are shown in Figure 8.1.

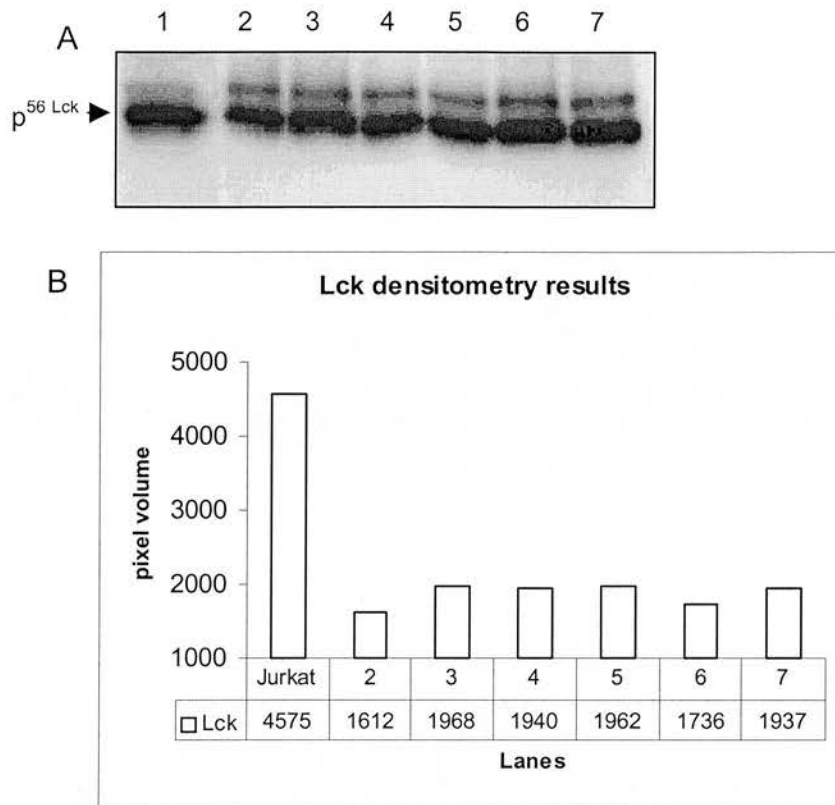


Figure 8. 1: p56^{Lck} phosphorylation is not affected by BB-3103. (A) p56^{Lck} immunoblot of WT and CD62L deficient CD4⁺ T cells activated with 20µg/ml immobilised anti-CD3 mAb and BB-3103 for 1 minute. Lane 1-Jurkat whole cell lysate +ve control. Lane 2, WT no treatment control. Lane 3 WT 1 min anti-CD3 mAb. Lane 4 WT 1 min anti-CD3 mAb + 20µM BB-3103. Lane 5 CD62L deficient CD4⁺ T cells no treatment control. Lane 6 CD62L deficient CD4⁺ T cells 1 min anti-CD3 mAb. Lane 7 CD62L deficient CD4⁺ T cells 1 min anti-CD3 mAb + 20µM BB-3103. (B) Results of densitometry performed on Lck blot using GrabIt/GelBase software (UVP, Cambridge, U.K.).

Although strong signals for phosphorylated p56^{Lck} were detected in all lanes, no differences were observed between samples tested. Furthermore, there was no detectable difference between resting cells and cells activated with immobilised anti-CD3 mAb for 1 minute. Soluble monoclonal anti-CD28 mAb was not added to the system since early time points were being examined and evidence showed CD28 signalling helps maintain long term phosphorylation of signal transducing molecules (Lu *et al.* 1994). Recent evidence, however, showed secondary regulatory pathways in the activation of p56^{Lck} is determined by

factors other than phosphorylation, namely CD28 regulation of p56^{lck} (Holdorf *et al.* 1999). Also, it is possible that the immunoblotting procedure may not have been sensitive enough to detect minor changes in phosphorylation patterns of p56^{lck}. Jurkat cells were not activated with anti-human CD3 ζ mAb since this would lead to apoptosis (Ruiz-Ruiz *et al.* 1995; Zhu and Anasetti 1995). PY-99 and rabbit anti p56^{lck} antibodies are species cross reactive, thus reacting with human Jurkat cells as well as murine CD4⁺ T cells.

SUMMARY

- BB-3103 had no detectable effect on Lck tyrosine phosphorylation.

8.1.2 ITAM PHOSPHORYLATION ON CD3 ζ IS MODIFIED BY BB-3103

ITAM phosphorylation patterns on CD3 ζ subunit of the TCR complex determines the activation state of the CD4⁺ T cell and subsequently the fate of the T cell (Kersh *et al.* 1998; Madrenas *et al.* 1995; Sloan-Lancaster *et al.* 1994). The experiment below describes the detection of modified ITAM phosphorylation pattern of CD3 ζ in the presence of BB-3103 in purified WT and CD62L deficient CD4⁺ T cells. The lysates were immunoprecipitated as before (see p56^{Lck}) but immunoblotted with anti-CD3 ζ mAb (NH₂-specificity mouse and human reactive) and results are shown in Figure 8.2.

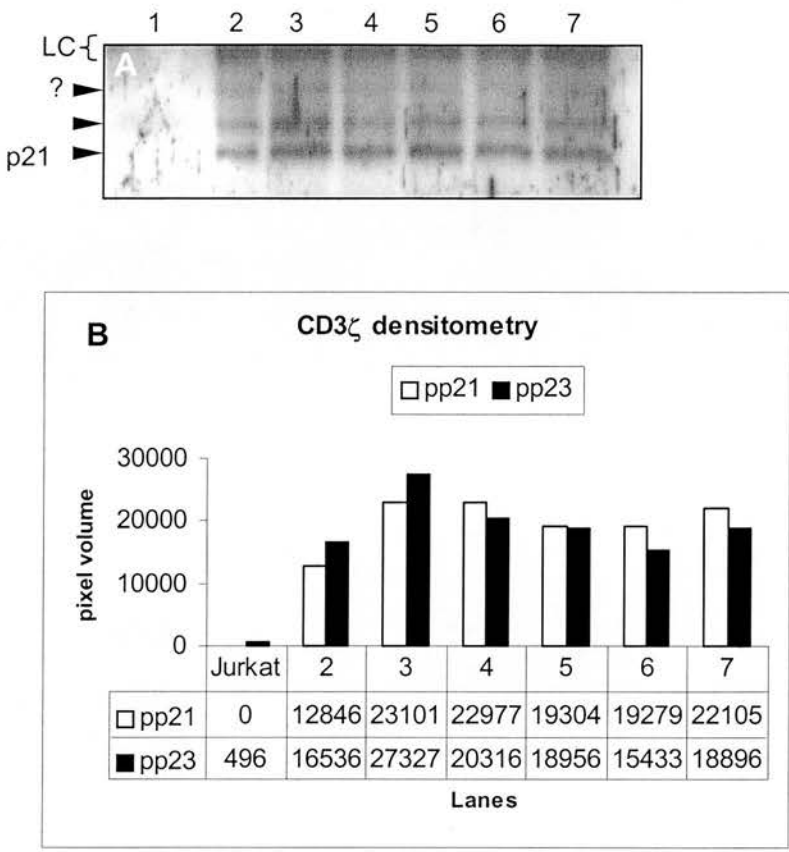


Figure 8. 2: ITAM phosphorylation on CD3ζ is modified by BB-3103.(A) CD3ζ immunoblot of WT and CD62L deficient CD4⁺ T cells activated with 20μg/ml immobilised anti-CD3 mAb and BB-3103. Lane 1-Jurkat whole cell lysate +ve control. Lane 2, WT no treatment control. Lane 3 WT 1 min anti-CD3 mAb. Lane 4 WT 1 min Anti-CD3 mAb+ 20μM BB-3103. Lane 5 CD62L deficient no treatment control. Lane 6 CD62L deficient 1 min anti-CD3 mAb. Lane 7 CD62L deficient 1 min anti-CD3 mAb+ 20μM BB-3103. Equal loading of protein was assessed by density of PY99 light chain (LC) at 25kDa. (B) Results of densitometry performed on CD3ζ blot using GrabIt/GelBase software (UVP, Cambridge, U.K.).

The Jurkat positive control contains only one phospho-isoform of CD3ζ - p23 (Figure 8.2). The WT control, however, contains both phospho-isoforms (p21, p23) of CD3ζ suggesting a state of slight activation perhaps due to cell isolation and centrifugations. After one minute of exposure to immobilised anti-CD3 mAb, WT CD4⁺ T cells upregulated both the p21 and p23

CD3 ζ phospho-isoforms. In the presence of BB-3103, however, only p21 was visibly upregulated suggesting a variation in CD3 ζ phosphorylation and, thus, an altered activation state as compared to no inhibitor control. The CD62L deficient CD4⁺ T cells, on the other hand, did not upregulate any phospho-isoforms of CD3 ζ upon stimulation and this pattern was not altered in the presence of BB-3103. These results suggest that inhibition of T cell proliferation by BB-3103 correlates with a change in CD3 ζ chain phosphorylation, and this effect was not observed in the CD62L deficient mice.

SUMMARY

- Presence of BB-3103 seemed to reduced production of p23 isoform of CD3 ζ in WT CD4⁺ T cells, whereas it had no significant effect on CD62L deficient CD4⁺ T cells. These data however, cannot accurately be quantified using this method of detection (see discussion).
- CD62L deficient T cells did not upregulate either phospho-isoforms in the time points investigated. These data however, cannot accurately be quantified using this method of detection (see discussion).

8.1.3 ZAP-70 PHOSPHORYLATION IS REDUCED BY BB-3103

ZAP-70 (ζ associated protein 70kDa) is the downstream transducer molecule from CD3 phosphorylation which associates with phosphorylated ITAMs on the ζ subunit of CD3 (Chan *et al.* 1994b). Upon docking with correctly phosphorylated ITAM sequences, ZAP-70 undergoes activation by phosphorylation of Tyrosine residues mediated by Lck (Iwashima *et al.* 1994; Weil *et al.* 1995). The next experiments were designed to investigate the effect of BB-3103 on the phosphorylation state of ZAP-70 after stimulation with immobilised anti CD3 ϵ monoclonal antibody. Figure 8.3 shows the ZAP-70 immunoblot performed on WT CD4⁺ T cells in the presence of BB-3103. Samples were treated as described in the legend and immunoprecipitated with mouse monoclonal anti-phospho-Tyrosine (PY-20), electrophoresed through 12% SDS-PAGE and transferred to nitrocellulose. Blots were probed with rabbit monoclonal anti-ZAP-70 antibody and goat anti-rabbit HRP conjugated mAb used as detection antibody.

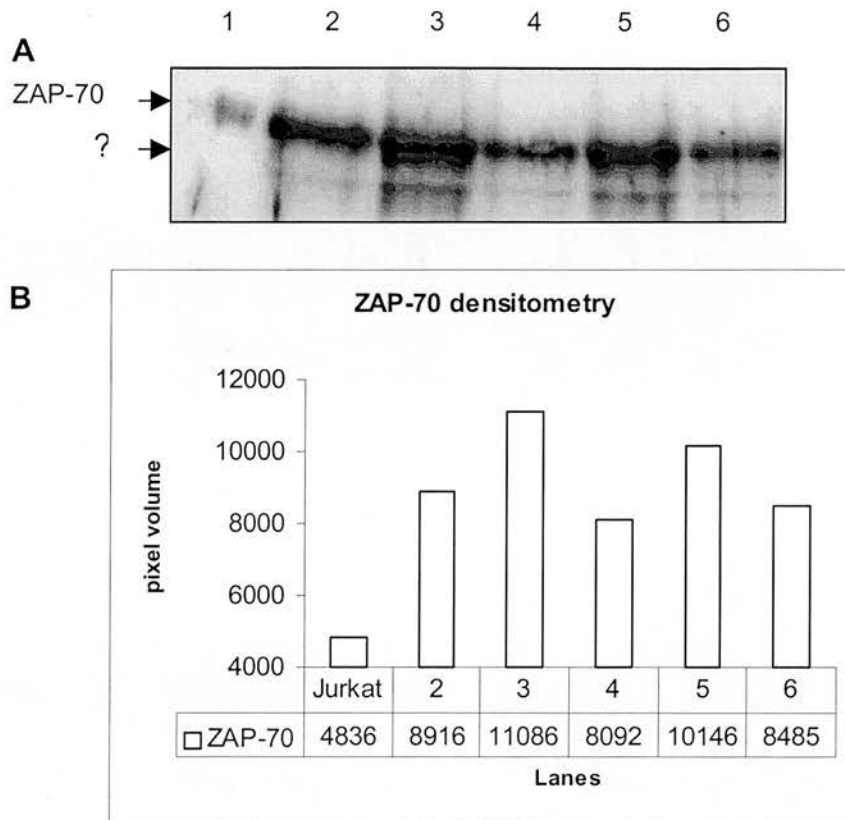


Figure 8. 3: ZAP-70 phosphorylation is modified by BB-3103. ZAP-70 immunoblot of WT CD4⁺ T cells activated with 20µg/ml immobilised anti-CD3 mAb in the presence or absence of BB-3103. Lane 1, Jurkat whole cell lysate (+ve control). Lane 2, freshly purified CD4⁺ T cells. Lane 3, 1 minute activation control. Lane 4, 1 minute activation + 20µM BB-3103. Lane 5, 5 minute activation control. Lane 6, 5 minute activation +20µM BB-3103.

ZAP-70 phosphorylation was inhibited in the presence of BB-3103 (Figure 8.3). These results demonstrate that treatment of WT CD4⁺ T cells with BB-3103 can directly affect intracellular signalling by altering tyrosine phosphorylation of key signalling molecules such as CD3ζ and ZAP-70. The consequence of these altered signalling patterns is potentially the causative factor in decreasing T cell activation and subsequent IL-2 dependent proliferation.

SUMMARY

- Presence of BB-3103 upon activation of CD4⁺ T cells reduced ZAP-70 phosphorylation. These data however, cannot accurately be quantified using this method of detection (see discussion)

8.2 CALCIUM FLUX IN T CELLS IS MODIFIED BY BB-3103

The signalling pathways which lead to T cell activation and subsequent proliferation depend on MAPK pathways as well as calcineurin activation. Calcineurin is a calcium dependent signal transducing molecule which complements the MAPK signalling cascade (Cantrell 1996) (Figure 1.11). The two pathways are independent but both are required to complete T cell activation and subsequent proliferation (Radvanyi *et al.* 1996).

8.2.1 BB-3103 AFFECTS HUMAN CD4⁺ T CELL PROLIFERATION

The model used for calcium flux in this project used Fura-2AM loaded human CD4⁺ T cells in a stirred cell suspension system, enabling results to represent the flux of 10×10^6 cells per sample. Human CD4⁺ T cells were used for these experiments due to the availability of anti-CD3 monoclonal antibodies capable of activating T cells in soluble form (i.e. HIT3a and OKT3 mAb). No soluble, mitogenic mAbs against murine CD3 were available at the time of the experiments. The effect of BB-3103 on human CD3⁺ T cell proliferation isolated from peripheral blood by MACS, was similar to the effect seen on murine CD4⁺ T cells (Figure 8.4).

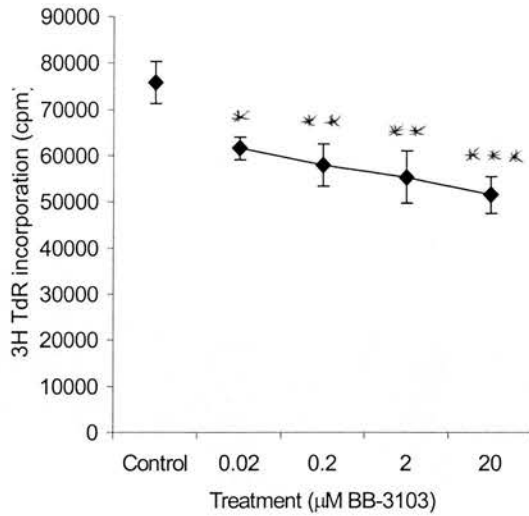


Figure 8. 4: Proliferation of human CD3⁺ cells is inhibited by BB-3103. Graph showing the effect of BB-3103 on proliferating human CD3⁺ T cells using immobilised anti-CD3 mAb and 5μg/ml soluble anti-CD28 mAb for 3 days. Graph represents mean of three experiments performed on 3 separate individuals. Proliferation was assessed by thymidine incorporation for the last 6hrs in culture. Background proliferation did not exceed 5000 cpm.

Human T cell proliferation was also inhibited in the presence of BB-3103 suggesting the mechanism of murine CD4⁺ T cell inhibition may be similar in human T cells (compare Figures 3.7 and 8.4).

8.2.2 BB-3103 INCREASES TCR MEDIATED $[Ca^{2+}]_i$ FLUX

The effect of incubating human CD4⁺ T cells with 20 μ M BB-3103 prior to addition of HIT3a mAb (Figure 8.5).

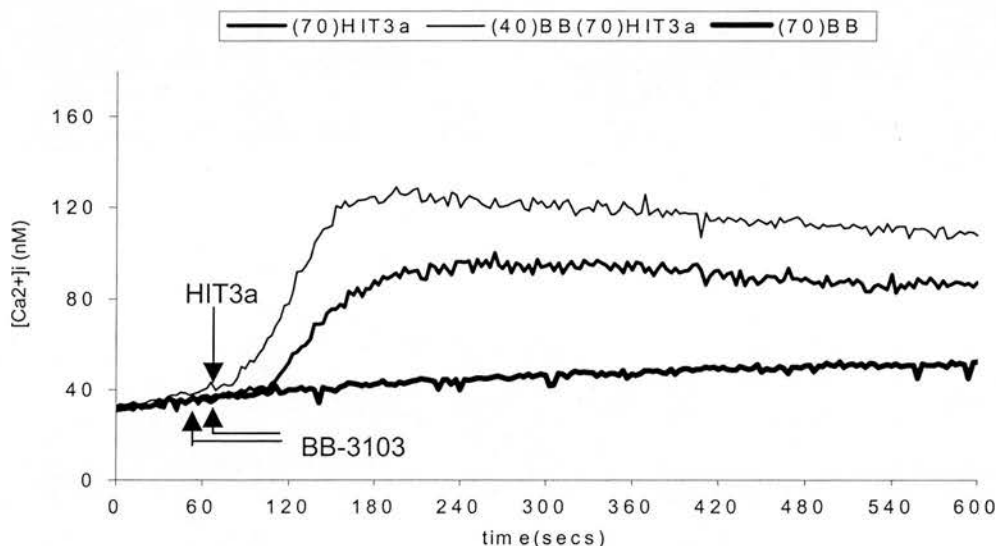


Figure 8. 5: BB-3103 increases TCR mediated $[Ca^{2+}]_i$ flux. Graph representing calcium flux in human CD4⁺ T cells resuspended in Ca^{2+} containing medium in the presence or absence of 20 μ M BB-3103. Thick line shows calcium flux in cells treated with 20 μ M BB-3103 only at 70 seconds. Medium thick line depicts the calcium flux of cells treated with 10 μ g/ml HIT3a at 70 seconds. Thin line shows calcium flux in T cells pre-incubated with 20 μ M BB-3103 (added at 40secs) and activated with 10 μ g/ml HIT3a mAb at 70 seconds. Graph shows results from one experiment representative of three experiments performed on cells isolated from 3 individuals.

The highest dose of BB-3103 (20 μ M) used had no effect on calcium flux in the human CD4⁺ T cells when added alone (Figure 8.5). Interestingly, the pre-incubation of T cells with 20 μ M BB-3103 prior to HIT3a addition induced a more rapid and augmented calcium flux within the CD4⁺ T cells compared to the flux observed in HIT3a only samples. The increase in Ca^{2+} concentration was maintained for 10 minutes and was constantly higher in the BB-3103 treated groups.

8.2.3 BB-3103 INCREASES CD62L MEDIATED [Ca²⁺]_i FLUX

The following experiments investigated the role of cross-linking CD62L in inducing calcium flux within human CD4⁺ T cells and the effect of adding BB-3103 prior to cross-linking CD62L (Figure 8.6).

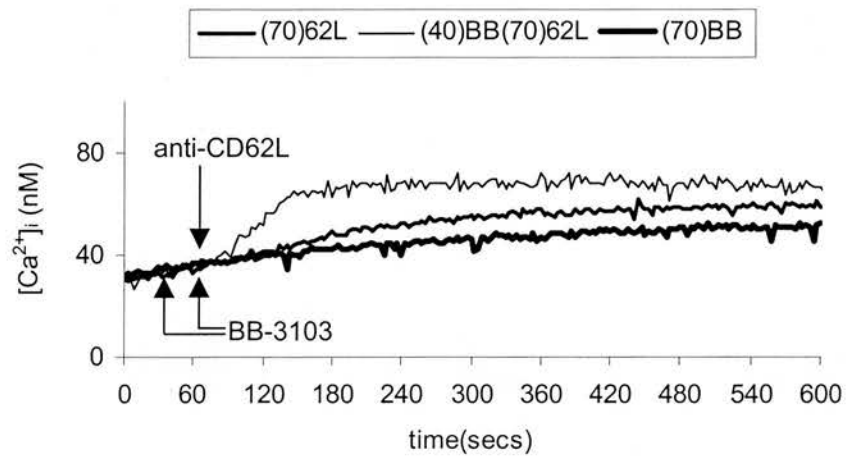


Figure 8. 6: BB-3103 increases CD62L mediated [Ca²⁺]_i flux. Graph representing the calcium flux within human CD4⁺ T cells treated with 10µg/ml anti-CD62L mAb in the presence or the absence of BB-3103. Thick line represents control cells treated with 20µM BB-3103 at 70 seconds. Medium thick line represents cells treated with 10µg/ml anti-CD62L mAb at 70 seconds thus inducing the shedding from the surface of the cell. Thin line represents data observed from cells treated with 20µM BB-3103 (40 seconds) prior to addition of 10µg/ml anti-CD62L (70 seconds). Graph shows results from one experiment representative of three consecutive experiments performed on cells isolated from 3 individuals.

Antibody mediated cross-linking CD62L on the surface of a CD4⁺ T cell induced a calcium flux. The increase in calcium concentration in the cells however, is greater in the presence of inhibitor suggesting that maintaining the cross-linked CD62L on the surface of the cell could enhance the calcium signalling pathway.

SUMMARY

- The addition of BB-3103:
 - on human CD4⁺ T cells activation induced a rapid and augmented calcium flux and
 - upon cross-linking CD62L with monoclonal antibody induces an increase in calcium flux independently from any TCR mediated signalling.

DISCUSSION (CHAPTER 8)

For phosphorylation experiments in this chapter, Western blots were developed using enhanced chemiluminescence (ECL) and developed using light sensitive X-Ray film. It should be noted that quantitative analysis of films developed in this fashion is highly inaccurate due to the limitations of the X-Ray film. The films used in these experiments have a finite detection level with a plateau, beyond which detection is highly inaccurate. In order to validate phosphorylation quantitatively, the Western blots should have been developed using phospho-imaging apparatus which does not plateau and therefore, poses no risk in quantitating over-exposed films. The densitometry shown in this chapter, therefore, should not be over-interpreted as these experiments should be repeated using a phospho-imager.

This section described that BB-3103 was capable of modifying signalling pathways upon activation of T cells through the TCR. As described in Figure 1.11 one of the first events upon TCR engagement, is the activation of the Src tyrosine kinase Lck (Cantrell 1996). The method used in this study may not have been sensitive enough to detect changes in Lck activation status. Also, the signalling experiments were performed in the absence of soluble

anti-CD28 mAb, but since recent evidence describes CD28 involvement in regulation of Lck (Holdorf *et al.* 1999), the experiments could be repeated in the presence of anti-CD28 mAb.

Following Lck activation, CD3 ζ ITAM phosphorylation occurs (Cantrell 1996) and generates two CD3 ζ phospho-isoforms, pp21 and pp23. Activation of wild type CD4⁺ T cells with immobilised anti-CD3 ϵ monoclonal antibodies appeared to upregulate both isoforms as previously described (Kersh *et al.* 1998). In the presence of BB-3103 however, the upregulation of pp23 seemed to be reduced suggesting the possible generation of different phosphorylation patterns. CD62L deficient CD4⁺ T cells did not seem to upregulate either phospho-isoforms at the time points investigated and there seemed to be no variation in phosphorylation was observed in the presence of BB-3103. This would suggest that CD62L may modulate CD3 ζ phosphorylation and that inhibition of CD62L shedding by BB-3103 could possibly downregulate the capability of CD62L to aid CD3 ζ signalling.

Following phosphorylation of CD3 ζ ITAM sequences, Syk tyrosine kinase ZAP-70 docks to the phospho ITAMs and becomes tyrosine phosphorylated itself by Lck (Cantrell 1996). Activation of ZAP-70 was also investigated and suggested that CD4⁺ T cells in the presence of BB-3103, significantly less ZAP-70 was found in the activated form using standard ECL detection methods. These results, and suggestions of modified CD3 ζ phosphorylation, suggest that ZAP-70 may not subsequently be activated.

Since CD62L has been immunoprecipitated with several different signalling molecules (CD3 complex (Murakawa *et al.* 1992), Lck (Brenner *et al.* 1996) and Calmodulin (CaM) (Kahn *et al.* 1998), it is possible that shedding of CD62L enhances activation of Lck by a dissociation mechanism, thus leading to reduced or retarded release of Lck when shedding of CD62L is inhibited. Downregulation of ZAP-70 activation would subsequently lead to reduced IL-2 secretion which was observed in proliferation assays in the presence of BB-3103.

CD69 expression, however, was reported to be regulated by Ras activation (D'Ambrosio *et al.* 1994), which is an integral part of the TCR activation pathway (Figure 1.11). Based on the results of signalling observations reported in this thesis, CD69 expression should be downregulated, however, this is not the case and may suggest that CD69 expression may be regulated through another pathway or that the Ras activation in the presence of BB-3103 is sufficient for the upregulation of CD69.

Calcium studies revealed that in the presence of BB-3103, a rapid and elevated flux in calcium ions was observed. In the absence of BB-3103, however, a 40 second delay was observed between activation through the TCR and the detection of a reduced calcium flux, presumably due to the step-wise activation of molecules (ZAP-70, LAT etc.), eventually leading to the increase in calcium mobilisation. When pre-incubated with BB-3103, T cells demonstrated very little delay between TCR activation and calcium mobilisation (15 seconds). This observation may be linked to the association of calmodulin with the cytoplasmic tail of CD62L (Kahn *et al.* 1998), where upon TCR activation, intracellular serine phosphorylation of the CD62L cytoplasmic tail initiates dissociation of calmodulin and subsequent shedding of CD62L. Inhibiting CD62L shedding may either enhance activation induced serine phosphorylation, thereby, leading to more calmodulin dissociation, or cause CD62L to maintain a conformational structure which promotes activation of calmodulin.

In separate observations, it was established that the mechanism by which the calcium flux was increased upon TCR activation may have been due to CD62L being maintained on the surface of the cell since cross-linking CD62L with monoclonal antibodies demonstrated an increased calcium flux in the presence of BB-3103.

Figure 8.7 hypothesises a possible mechanism by which inhibition of CD62L shedding could downregulate tyrosine phosphorylation whilst upregulating calcium mobilisation.

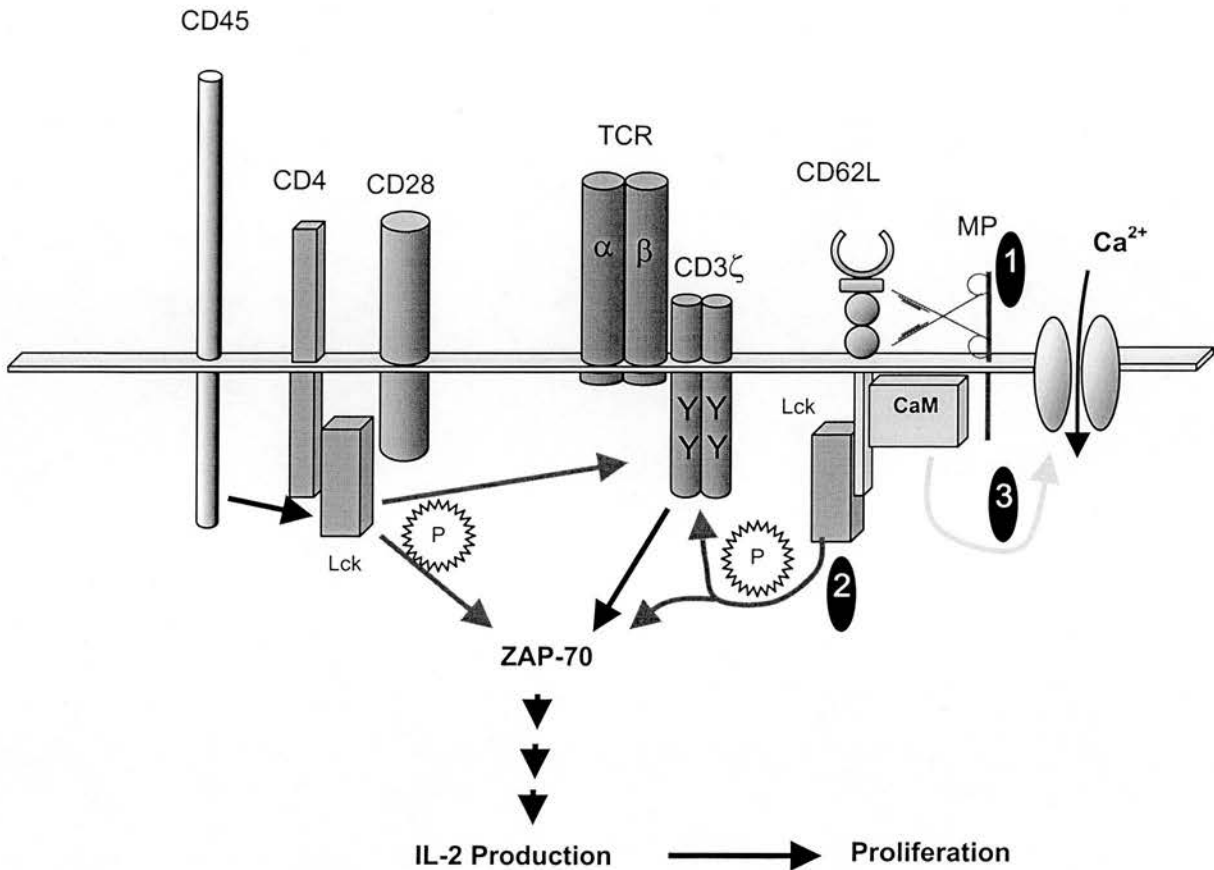


Figure 8. 7: Diagrammatic representation of possible involvement of CD62L in the regulation of T cell activation. Upon normal activation, Lck is activated through CD45 phosphatase and the release from CD62L cytoplasmic tail leading to CD3ζ ITAM phosphorylation. ZAP-70 becomes activated and through several signalling pathways, leads to IL-2 production. In the presence of BB-3103, however, it is possible to speculate that Lck activation is decreased since CD62L is not shed (①) and does not release Lck, this event leads to modified or reduced ITAM phosphorylation (②) which subsequently leads to decreased IL-2 production. Inhibition of CD62L shedding may induce enhanced calmodulin release by unknown mechanisms (③) which could lead to a more rapid and elevated calcium flux. CaM=Calmodulin.

CHAPTER 9

9.1 BB-3103 DIFFERENTIALLY AFFECTS HUMAN Th₁ AND Th₂ CD4⁺ T CELL SUBSETS

Upon activation, naïve CD4⁺ T cells adopt an effector phenotype and can differentiate into various T helper subsets called Th₀, Th₁ or Th₂ (Mosmann *et al.* 1986; Romagnani 1994). This functional categorisation is based on the cytokines that the T cells secrete and is determined by various factors described in the introduction of this thesis (Section 1.7). The results presented here demonstrate that CD62L plays a critical role in regulating T cell activation. Recent reports state that CD62L expression, originally thought to be maintained on naïve CD4⁺ T cells only, is in fact maintained on Th₁ cells (Wely *et al.* 1999). The next set of experiments, therefore, were designed to investigate any potential differential effects of BB-3103 on the two CD4⁺ T helper subsets, Th₁ and Th₂.

Human subjects were chosen according to their atopic status to provide Th₁ and Th₂ CD4⁺ responses. Six patients with acute atopic dermatitis were provided by Dr Bernadette DaSilva from the Dermatology Dept (Royal Infirmary of Edinburgh) for the CD4⁺ Th₂ type T cell response. Atopic patients were selected according to positive tests for skin test reaction against house dust mite allergens and high serum IgE antibody titres (results not shown). Six non-atopic volunteers with no history of allergy or asthma were selected for the Th₁ type CD4⁺ T cell response. Non atopic volunteers showed normal serum IgE antibody titres (results not shown).

9.1.1 CYTOKINE SECRETION FROM PROLIFERATING ATOPIC AND CONTROL PBMCs TO HOUSE DUST MITE AEROALLERGEN Der p 1

Experiments were designed to establish the cytokine secretion pattern of human T cells to the common aeroallergen derived from house dust mite *Dermatophagoides pteronyssinus*, Der p 1. Whole PBMCs from 2 atopic and 2 non-atopic patients were isolated and cultured for 48hrs in the presence of 10 μ g/ml Der p 1. Supernatants were collected at 48hrs and concentrations of IFN γ , IL-2, IL-4, IL-5 and IL-12 determined (Figure 9.1). No IL-12 was detected (results not shown) and the concentrations of IL-5 may be misleading since they are at the limits of detection for this assay.

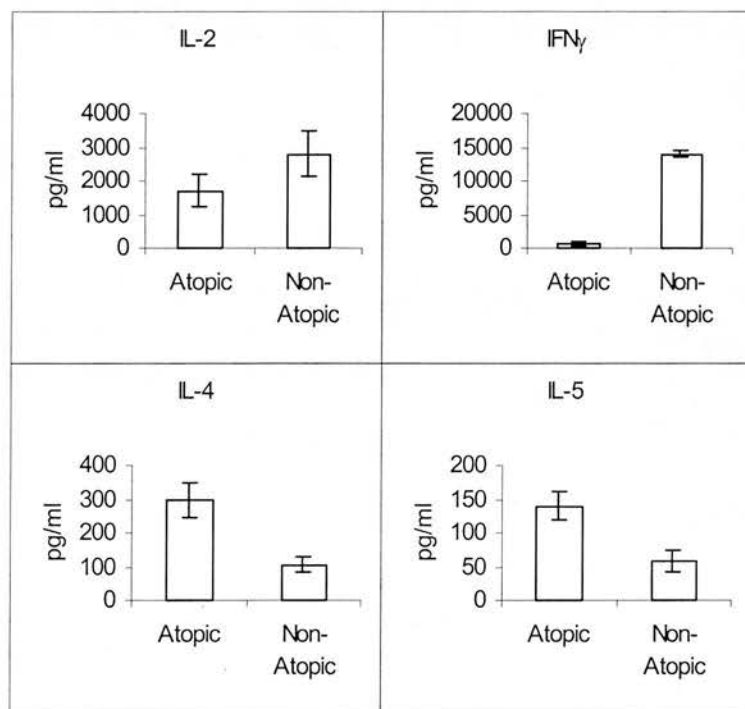


Figure 9. 1: Cytokine secretion from proliferating atopic and non-atopic PBMCs to Der p 1. Cytokine secretion at 48hrs from atopic and non-atopic PBMCs stimulated with 10 μ g/ml Der p 1. IL-5 lower detection limit was 50ng/ml therefore, the non-atopic values may not accurately depict actual concentrations of IL-5 in culture supernatant.

Cytokine secretion reveals that proliferating T cells from atopic PBMCs secrete a Th₂ like cytokine profile (high IL-4 and IL-5, low IL-2 and IFN γ). T cells from non atopic patients

secrete a cytokine profile characterising a Th1 type response (high IL-2 and IFN γ with low IL-4 and IL-5) (Figure 9.1).

9.1.2 NON-ATOPIC PBMC PROLIFERATION TO Der p 1 IN THE PRESENCE OF BB-3103

Whole PBMC populations from atopic and non atopic patients were used in proliferation assays. To investigate the effect of BB-3103 further, the inhibitor was added at various times (t=0hrs, t=24hrs and t=48hrs) post antigen stimulation. A summary of the CD4⁺ T cell proliferation profile of these two patient groups in the presence of BB-3103 is presented in Figures 9.2 and 9.3. The raw data for all the patients are presented in the appendix at the end of this thesis.

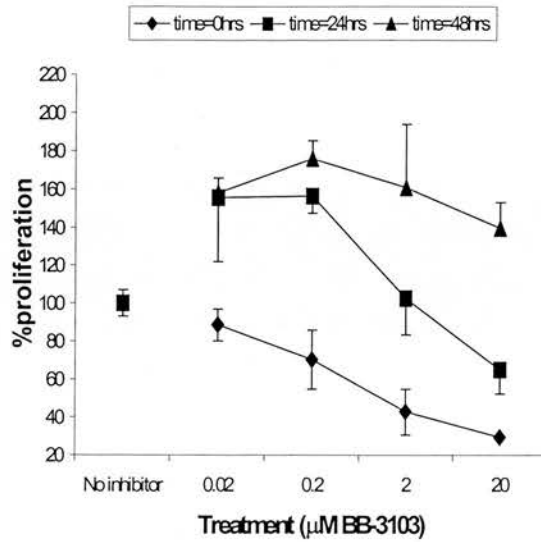


Figure 9. 2: Non-atopic PBMC proliferation to Der p 1 in the presence of BB-3103. Graph representing a summary of the CD4⁺ T cell proliferation of PBMCs isolated from six non-atopic patients (Th₁ response) to 10μg/ml Der p 1 in the presence of BB-3103. Data presented as percentage of control proliferation to normalise all patients.

The effect of BB-3103 on cells isolated from non-atopic patients reveals some striking findings. When the inhibitor was added at t=0hrs, the expected decrease in proliferation was observed, however, when the inhibitor was added at later time points (t=24 and t=48hrs) the proliferation was markedly increased.

9.1.3 ATOPIC PBMC PROLIFERATION TO Der p 1 IN THE PRESENCE OF BB-3103

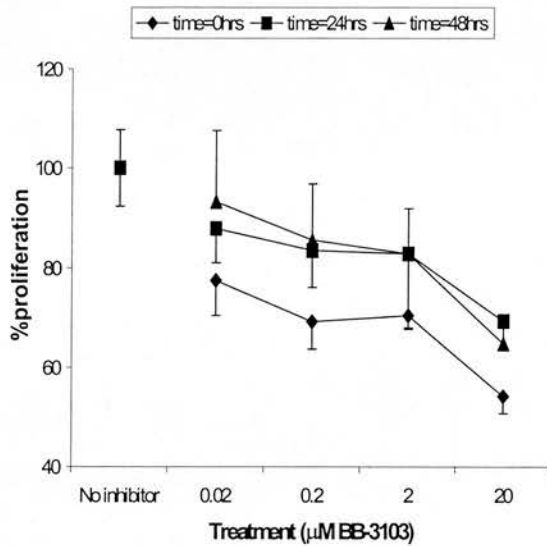


Figure 9. 3: Atopic PBMC proliferation to Der p 1 in the presence of BB-3103. Graph representing a summary of the CD4⁺ T cell proliferation of PBMCs from six atopic patients to 10μg/ml Der p1 in the presence of BB-3103. Data presented as percentage of control proliferation to normalise all patients.

Proliferation of cells isolated from atopic patients, as expected, was reduced by the addition of BB-3103 inhibitor at the initiation of the culture (time=0hrs) (Figure 9.3). In contrast to the non-atopic patients, addition of BB-3103 at later time points also decreased CD4⁺ T cell proliferation, although to a lesser extent than that observed at t=0hrs.

SUMMARY

- Proliferation of human PBMCs to 10µg/ml Der p 1 isolated from atopic and non-atopic patients revealed Th₂ and Th₁ type cytokine profiles respectively.
- Addition of BB-3103 to proliferation assays at the initiation of the cultures (t=0hrs) inhibited both atopic and non-atopic proliferative responses but at later time points (24 and 48hrs) enhanced Th₁ proliferation whereas Th₂ type responses were still inhibited.

9.1.4 MODIFIED CYTOKINE SECRETION FROM PBMCs IN THE PRESENCE OF BB-3103

The results represented in the two previous graphs demonstrate a differential effect of BB-3103 on proliferation of PBMCs isolated from atopic and non-atopic patients. These experiments were extended to investigate if the cytokine response profiles of the mixed populations isolated from atopic and non-atopic patients were differentially regulated by the addition of BB-3103. The cytokines analysed included IFN γ , IL-2, IL-4, IL-5 and IL-12 and were performed on the supernatants of bulk cultures from PBMCs isolated from two atopic and two non-atopic patients in the presence of 20 μ M BB-3103 only at 48 hrs. No IL-12 was detected in any supernatants (results not shown) and the concentrations of IL-5 may not accurately represent actual concentrations in culture supernatant since they lie at the lower limit of detection for this assay. The findings are represented in Figure 9.4.

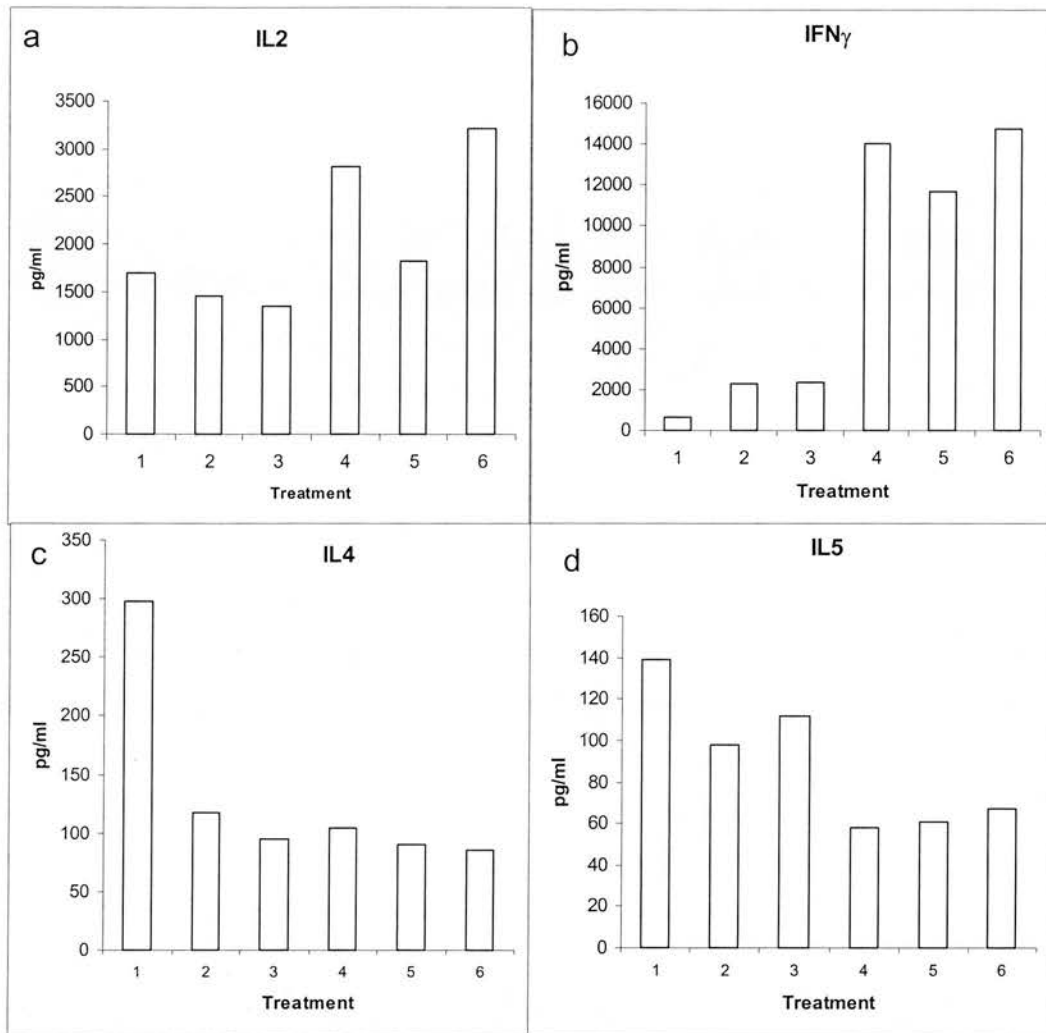


Figure 9. 4: Modified cytokine secretion from PBMCs in the presence of BB-3103. Cytokine secretion at 48 hrs from 2 atopic and 2 non-atopic PBMCs activated with 10 μ g/ml Der p 1 in the presence or absence of 20 μ M BB-3103 as shown on the x-axis. Panel (a) IL-2 concentrations, panel (b) IFN γ secretion, panel (c) IL-4 secretion, panel (d) IL-5 secretion. Lane 1, Atopic control; Lane 2, atopic with 20 μ M BB-3103 at 0hrs; Lane 3, atopic with 20 μ M BB-3103 at 24hrs; Lane 4, Non-atopic control; Lane 5, non-atopic with 20 μ M BB-3103 at 0hrs; Lane 6, non-atopic with 20 μ M BB-3103 at 24hrs. Lower detection limits of IL-5 ELISA was 50 ng/ml, therefore IL-5 concentration in non-atopics may not accurately represent the actual concentration of IL-5 present in the culture supernatant.

The levels of IL-2, predominantly secreted by Th₁ type T cells (Casolaro *et al.* 1995), in the control samples (i.e. in the absence BB-3103) was lower in atopic as compared to T cells from non-atopic individuals. Addition of BB-3103 at later time points to samples from atopic patients decreased the levels of IL-2 secretion. Addition of BB-3103 to T cell cultures from non-atopic individuals at 0 hours also decreased IL-2 concentration but surprisingly, had no effect on IL-2 production when added at an even later time point (24hrs). This finding parallels the proliferation results observed on the non-atopic PBMCs since the expansion of these cells was not inhibited by the presence of BB-3103 at later time points (24hrs and 48hrs).

IFN γ , a cytokine secreted by Th₁ and Th₀ cells (Oriss *et al.* 1997), was present at low levels in atopic controls and high levels in non-atopic controls, and showed a modest increase in atopic samples in the presence of BB-3103. This observation suggested that Th₂ CD4⁺ T cells secreted higher levels of IFN γ in the presence of BB-3103. Addition of BB-3103 to non-atopic samples at time 0hrs, decreased the secretion of IFN γ whereas BB-3103 had no effect on IFN γ levels when added at later time points.

IL-4, a cytokine secreted by Th₂ cells (Walker *et al.* 1992), on the other hand, was decreased in both atopic and non-atopic groups in the presence of BB-3103, regardless of time of addition to the samples. IL-4 is a strong Th₂ CD4⁺ T cell promoter and the presence of BB-3103 which downregulated the secretion pattern of IL-4 could therefore inhibit the promotion of Th₂ CD4⁺ T cell activation and differentiation.

IL-5, also a Th₂ cytokine (Lee *et al.* 1998) was present in higher concentrations in the atopic controls compared to non-atopic samples. The presence of BB-3103 in atopic samples, downregulated the secretion of IL-5 and had no effect on IL-5 secretion in non-atopic

samples. Again, the data suggested that BB-3103 was downregulating Th2 type cytokine secretion.

SUMMARY

- BB-3103 decreased levels of IL-2 in supernatants of T cells isolated from non-atopic individuals when added at 0hrs but had no effect when added at 24hrs
- BB-3103 increased levels of IFN γ in atopic supernatants when added at 0 and 24hrs but only decreased IFN γ levels in T cells from non-atopic patients when added at 0hrs
- IL-4 levels were markedly reduced in supernatants of T cells from atopic patients when BB-3103 was added at 0 and 24hrs.
- IL-5 levels were slightly decreased in the presence of BB-3103 at 0 hrs in the supernatants of T cells from atopic individuals.

9.2 BB-3103 DIFFERENTIALLY AFFECTS HUMAN TH₀ AND TH₂ CLONES

To confirm the results observed in the PBMCs from atopic and non-atopic patients, the effect of BB-3103 on established polarised human CD4⁺ T cell clones was analysed. The clones investigated were HA1.7 and AC1.1. HA1.7 is a Th₀ clone specific for peptide 306-318 from influenza virus haemagglutinin (HA) (Eckels *et al.* 1984; Lamb *et al.* 1982). AC1.1 is a Th₂ CD4⁺ T cell clone specific for peptide (28-40) in *Dermatophagoides pteronyssinus* group 2 allergen, Der p 2 (Verhoef *et al.* 1993).

T cell clones were stimulated with irradiated histocompatible APC (PBMCs) and the optimal peptide concentration (1.0 µg/ml) for 3 days in the presence or absence of BB-3103. The results are shown in Figure 9.5.

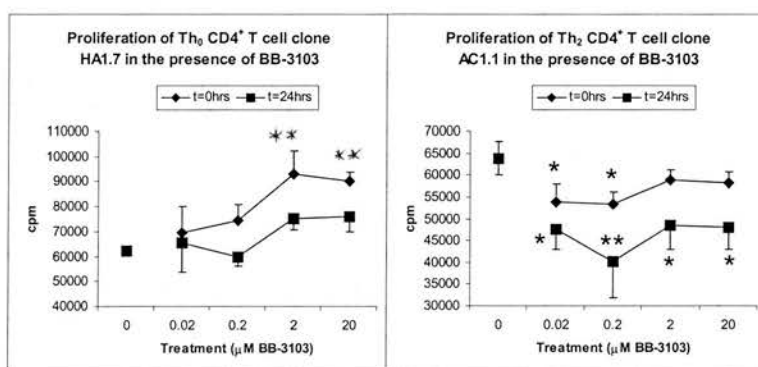


Figure 9. 5: BB-3103 differentially affects human Th₀ and Th₂ clones. Proliferation of Th₀ and Th₂ clones (HA1.7 and AC1.1 respectively) in the presence of BB-3103. BB-3103 increases the proliferation of HA1.7 T cells, but decreases the proliferation of Th₂ cells. Experiment performed once. One way ANOVA statistics performed on individual triplicate samples within the same experiment.

The results from these preliminary experiments (Figure 9.5) further support the evidence for differential effects of BB-3103 on different T helper subsets. BB-3103, when added at later

time points enhanced the proliferation of the Th₀ clone but had the opposite effect on Th₂ type CD4⁺ T cells. One noticeable difference, however, was the lack of inhibition of proliferation altogether in HA1.7 (Th₀) when the inhibitor BB-3103 was added at time 0hrs.

SUMMARY

- BB-3103 reduced proliferation of Th₂ CD4⁺ human T cell clone AC1.1 although to a lesser extent than polyclonal CD4⁺ T cells.
- BB-3103 increased proliferation of human CD4⁺ Th₀ T cell clone HA1.7 with no inhibition of proliferation observed when added at 0hrs.

9.2.1 BB-3103 INDUCES IFN γ PRODUCTION IN Th₂ CD4⁺ CLONES

Cytokine secretion from the Th₀ and Th₂ cell clones was subsequently investigated and results are shown in Figure 9.6.

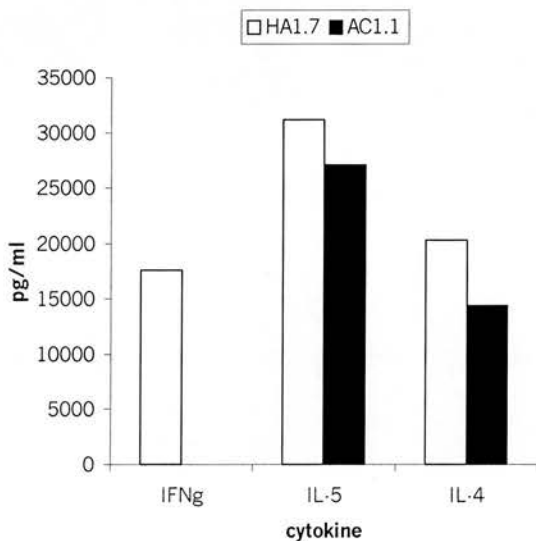


Figure 9. 6: Cytokine profile from AC1.1 and HA1.7 proliferations in the absence of BB-3103. Graphical representation of cytokine concentrations in supernatants from proliferation assays performed on HA1.7 and AC1.1 T cell clones in the absence of BB-3103. HA1.7, a Th₀ clone, secreted IFN γ , IL-5 and IL-4 cytokines, whereas AC1.1, a Th₂ clone, secreted IL-5 and IL-4, but no IFN γ .

Figure 9.6 shows the typical cytokine profile observed in HA1.7 and AC1.1 and these results verify the classification of these cells as Th₀-like (HA1.7) and Th₂-like (AC1.1). Cytokine secretion of HA1.7 and AC1.1 in the presence of BB-3103 was investigated (Figure 9.7).

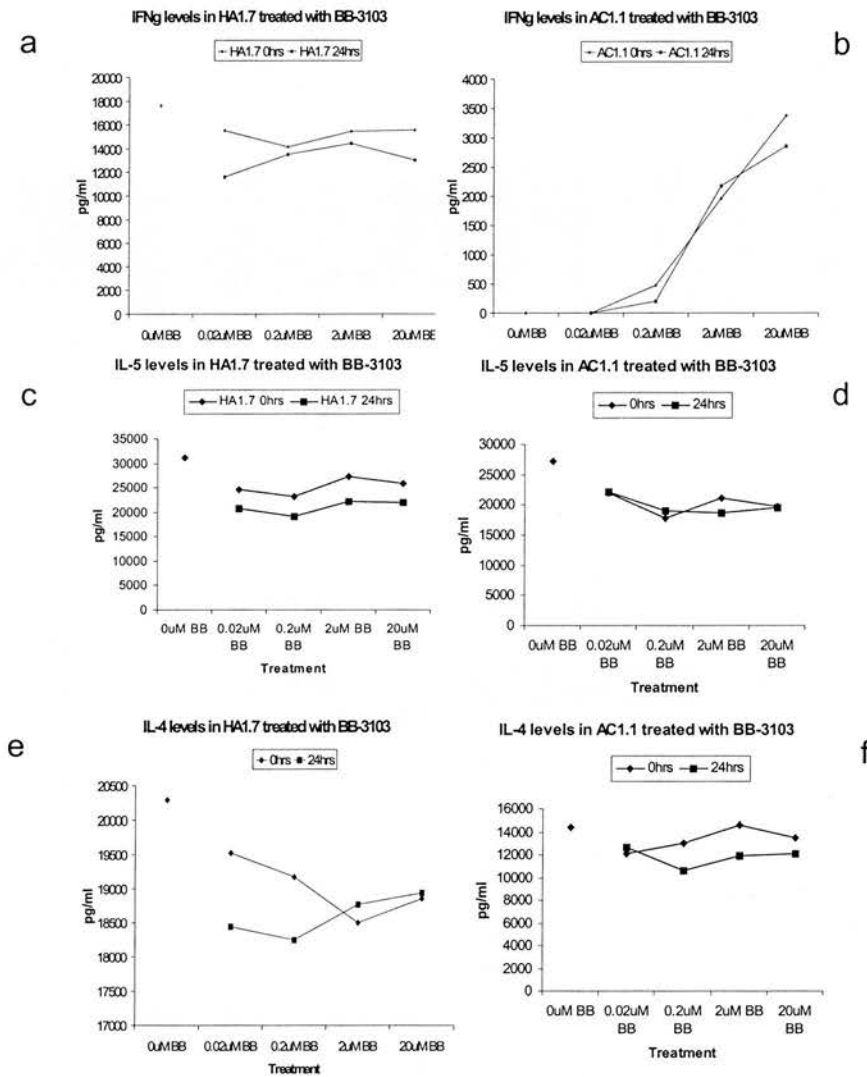


Figure 9. 7: BB-3103 induces IFN γ production in a human CD4⁺ Th₂ clone. Graphical representation of cytokines secreted from human CD4⁺ T cell clones HA1.7 (Th₀) and AC1.1 (Th₂) in the presence of BB-3103 added at various time points. Cytokines detected by sandwich ELISA. (a) IFN γ in HA1.7, (b) IFN γ in AC1.1, (c) IL-5 in HA1.7, (d) IL-5 in AC1.1, (e) IL-4 in HA1.7 and (f) IL-4 in AC1.1.

IFN γ secretion was not affected by BB-3103 in HA1.7 cells whereas, surprisingly, AC1.1 cells upon addition of BB-3103 started secreting IFN γ , suggesting a switch from Th₂ to either Th₀ or Th₁ type (Figure 9.7). IL-5 levels in both cell types were moderately decreased in the presence of BB-3103, consistent with the data obtained from the human PBMCs. IL-4 levels were strongly decreased in HA1.7 but unaffected in AC1.1 samples, which contradicts the data observed in the human PBMCs.

SUMMARY

- BB-3103 failed to alter Th₀ secretion of IFN γ but increased IFN γ secretion in cloned Th₂ CD4⁺ T cells when added at either 0 or 24hrs.
- BB-3103 moderately decreased IL-5 secretion in both Th₀ or Th₂ cell clones.
- BB-3103 reduced IL-4 production in Th₀ CD4⁺ T cell clone HA1.7 when added at either 0 or 24hrs, but had no effect on IL-4 production from AC1.1 cells.

9.3 BB-3103 AFFECTS MURINE POLARISED CD4⁺ T CELLS

9.3.1 INDUCTION OF Th₁ AND Th₂ TYPE ENVIRONMENT

Since the effector functions of different subsets of human T helper CD4⁺ cells were modulated by BB-3103, it was relevant to examine whether or not exogenous cytokine addition to activated murine naïve wild type CD4⁺ T cells could modify the pattern of responses in the presence of BB-3103. CD4⁺ T cells were isolated from unimmunised C57BL/6 spleens by MACS purification and stimulated in the presence of neutralising mAb and exogenous cytokines. To induce CD4⁺ T cells to adopt a Th₁ phenotype, 20µg/ml neutralising anti-IL-4 mAb (11B11 clone) as well as 20ng/ml recombinant murine IL-12 and 20ng/ml recombinant murine IFN γ , was added to the cultures at the time of stimulus with immobilised anti-CD3 mAb and 5µg/ml anti-CD28 mAb. For a Th₂ type phenotype, 20µg/ml neutralising IFN γ mAb (HB170 clone) and 20ng/ml recombinant murine IL-4 was added to the cultures at the time of stimulus with 5µg/ml anti-CD3 mAb and 5µg/ml anti-CD28 mAb. In Figure 9.8, the cytokine profile 48hrs-post stimulus of CD4⁺ T cells in the presence or absence of exogenous additions (see above) is presented.

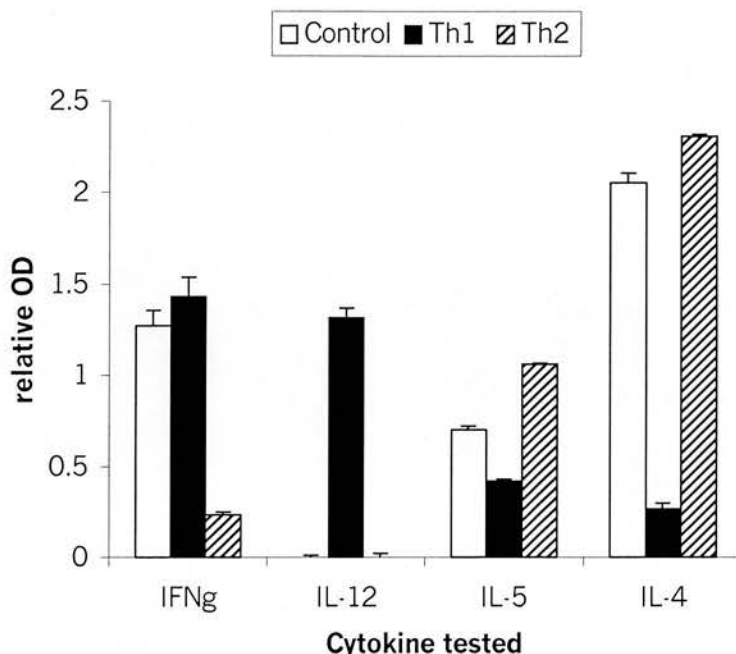


Figure 9. 8: Induction of a Th₁ or Th₂ type environment. Graph representing cytokine profile in purified CD4⁺ T cell stimulation in the presence of exogenous neutralising antibodies and recombinant cytokines. Control, no addition of exogenous cytokines. Th1-20μg/ml anti-IL-4 mAb, 20ng/ml rIL-12, 20ng/ml rIL-12, 20ng/ml rIL-12. Th2- 20μg/ml anti-IFN_γ mAb, 20ng/ml rIL-4. Graph represents one ELISA on pooled supernatants from 2 consecutive experiments.

The cytokine environment in which the CD4⁺ T cells are expanded is illustrated in Figure 9.8. Neutralising IFN_γ and IL-4 was successful in creating Th₂ and Th₁ environments, respectively. Since IL-5 was the only cytokine unmodified by addition of recombinant IL-5 or neutralising with anti-IL-5 antibodies, IL-5 is the only cytokine tested in these experiments which suggests that the protocol used was successful in generating Th₁ and Th₂ cells.

SUMMARY

- Addition of exogenous cytokines and neutralising antibodies to generate specific cytokine environments was successful in downregulating IL-5 for Th₁ and upregulating IL-5 for Th₂ cells.

9.3.2 PROLIFERATION OF MURINE Th₁ AND Th₂ CD4⁺ CELLS

In the next experiments, BB-3103 was added to both the Th₁ and Th₂ environments, and proliferation was assessed on day 3 by thymidine incorporation (Figure 9.9).

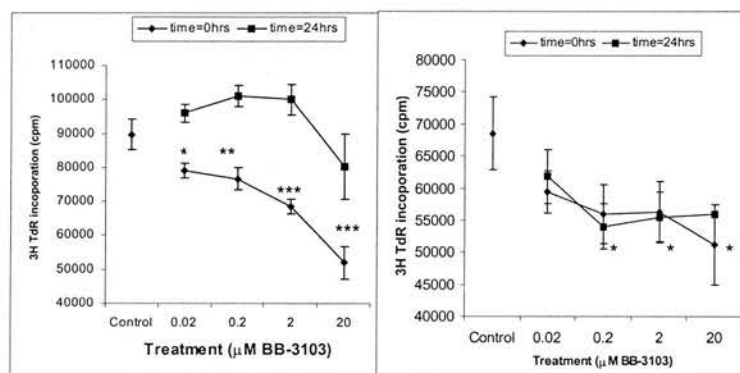


Figure 9. 9: Proliferation of murine Th₁ and Th₂ type CD4⁺ T cells. Graph representing the proliferation of purified naïve WT CD4⁺ T cells isolated from C57BL/6 spleens to immobilised anti-CD3 mAb and 5μg/ml soluble anti-CD28 mAb in the presence of either (a) Th₁ cytokines or (b) Th₂ cytokines and BB-3103. Addition of BB-3103 at time=0hrs inhibited proliferation of both Th₁ and Th₂ as seen before from the human PBMC data, whereas later addition (t=24hrs) induced an increase in proliferation of Th₁ cells but not Th₂ cells. Results represent the means of three animals. Background proliferation did not exceed 10000 cpm.

These observations demonstrate the importance of the local cytokine environment in determining the outcome of the proliferation of T cells in the presence of BB-3103 and supports the human PBMC proliferation to Der p 1 reported in sections 9.1.2 and 9.1.3. The level of proliferation in the controls of Th₁-like cultures was higher in all experiments as compared to the controls of Th₂ cells at equivalent cell numbers (89680cpm for Th₁ and 68547cpm for Th₂ cultures).

ELISAs were not performed on the experiments described above since addition of neutralising mAb and exogenous cytokines would not indicate any modification in cytokine secretion from the CD4⁺ T cells in the presence of BB-3103.

SUMMARY

- BB-3103 inhibited proliferation of both Th₁ and Th₂ CD4⁺ T cells when added at 0hrs.
- Th₁ type cells were unaffected by addition of BB-3103 at 24hrs, whereas Th₂ type cells were still inhibited by BB-3103 addition at 24hrs.

9.4 EXPRESSION OF CD62L IS MAINTAINED ON Th₁ TYPE CD4⁺ CELLS

The results obtained from the analysis of CD62L deficient CD4⁺ T cells suggested that the presence of CD62L on the cell surface was responsible for the decrease in proliferation by BB-3103. In addition, since Th₁ and Th₂ are differently affected by BB-3103, CD62L expression was investigated on T cells stimulated either in a Th₁ or Th₂ cytokine environment.

CD4⁺ T cells were isolated from the spleens of unimmunised C57BL/6 mice and stimulated with immobilised anti-CD3 mAb and 5µg/ml soluble anti-CD28 mAb in the appropriate cytokine/antibody environment to induce Th₁/Th₂ polarity as described before. Cell surface expression of CD62L was then determined on the CD4⁺ T cells 12hours after stimulation by flow cytometry (Figure 9.10).

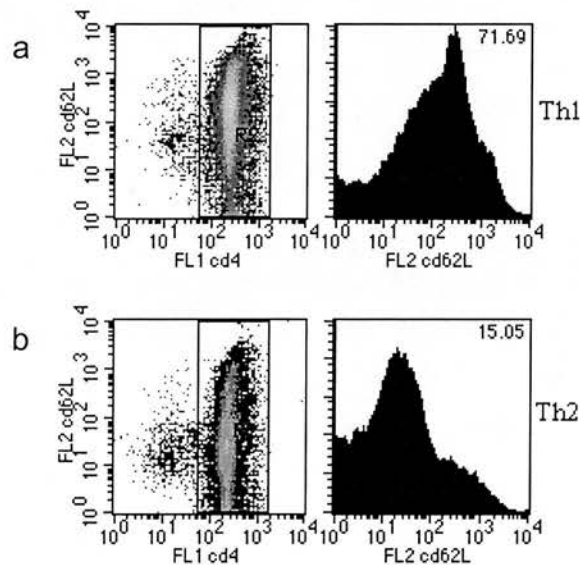


Figure 9. 10: CD62L expression is maintained on Th₁ CD4⁺ T cells. FACS profiles showing expression of CD62L on purified CD4⁺ T cells stimulated for 12hours in the presence of (a) 20µg/ml anti-IL-4 mAb, 20ng/ml IFN_γ and 20ng/ml IL-12 for Th₁ cells, and (b) 20µg/ml anti-IFN_γ mAb and 20ng/ml IL-4 for Th₂ cells. Mean fluorescent intensities are represented in the top right corner of the histograms. Experiment performed only once.

CD62L downregulation from activated CD4⁺ T cells is differentially regulated between Th₁ and Th₂ type CD4⁺ T cells in the absence of any metalloproteinase inhibitor (Figure 9.10). Since the presence of CD62L on the CD4⁺ T cell plays an important regulatory role in determining the activation state, the results suggest that BB-3103 has selected effects on Th₁ and Th₂ due to the differential CD62L expression patterns.

SUMMARY

- Th₁ type cells maintain CD62L expression on the surface upon activation, whereas Th₂ type cells shed CD62L.

DISCUSSION (CHAPTER 9)

PBMCs isolated from atopic and non-atopic patients revealed Th₂ and Th₁ type cytokine secretion as expected (Mosmann *et al.* 1986), but the phenotype of the resulting cells from these proliferation assays was not investigated. In order to establish what cell types proliferated after addition of Der p 1, flow cytometric analysis for T cell markers should have been performed to establish that T cells were indeed the cell type to be affected by BB-3103. The cells which proliferated from these assays could have been NK cells or $\gamma\delta$ T cells. If interpreted as $\alpha\beta$ TCR T cells proliferating, it could be assumed that, in proliferation assays, BB-3103 enhanced Th₁-type proliferation when added at 24hrs, whereas, Th₂-type proliferation was still inhibited at similar time points.

However, in proliferation assays investigating the effect of BB-3103 on the proliferation of human Th₀ and Th₂ clones, showed a Th₀ clone to be different since no inhibition occurred when BB-3103 was added at 0hrs which was not observed before. HA1.7 and AC1.1 were the only clones investigated, however, and may not have represented the expected functional heterogeneity. These experiments were only performed once.

Investigating differential effects of BB-3103 on Th₁ and Th₂ proliferation was further investigated by generating murine Th₁ and Th₂ CD4⁺ T cell types by addition of exogenous cytokine/neutralising antibody cocktails. In order to establish whether the correct cytokine profile had been generated, however, intracellular staining for cytokines should have been performed rather than ELISAs which were influenced by the exogenous recombinant cytokines added and the neutralising antibodies also present. From Figure 9.8, we can only assume that the cells generated under this protocol are Th₁ and Th₂ because of the IL-5 secretion pattern. Proliferation assays on these cells revealed that both Th₁ and Th₂ cells were inhibited when BB-3103 was added at early time points, but, like the non atopic human PBMC data, Th₁ cell proliferation was unaltered when BB-3103 was added at later time points.

Cytokine analysis of human Th₁ /Th₂-type CD4⁺ T cells in the presence of BB-3103 demonstrated a decrease in IL-2 secretion by Th₁ cells but no variation in IL-2 secretion in Th₂ cell types. Addition of BB-3103 at later time points increased IL-2 production in Th₁ cell types but still had no effect in Th₂ cell types. The IL-2 data obtained from Th₁ CD4⁺ T cells correlates with the proliferation data. Cytokines other than IL-2, were also modulated in culture supernatants from atopic and non-atopic individuals, namely IFN γ , IL-4 and IL-5. The response observed from in human PBMCs, however, is not restricted to the oversimplified 'polarised Th₁ and Th₂' paradigm, but rather a whole range of responses (Adorini *et al.* 1996) which could themselves be differentially modulated by BB-3103 and therefore give rise to complex cytokine pattern secretions.

Identification of cytokines secreted by human CD4⁺ T cell clones HA1.7 and AC1.1, revealed, as before very little modulation in pattern of secretion in the presence of BB-3103. These results however, should not be over interpreted as the clones were fed with irradiated

PBMC which may secrete vast amounts of cytokines following irradiation and/or could utilise cytokines secreted by the clones. This raises important issues which were not considered during the experiments as the donors which were used to feed the clones were not assayed for their atopy, which could strongly influence the outcome of cytokines secreted, not only by the T cells themselves but also by the irradiated PBMCs used as feeders. Taking this into account it appeared that AC1.1, the Th₂ type clone secreted IFN γ in increasing amounts correlating to BB-3103 concentration. This is the first identification that CD4⁺ Th₂ type cells could secrete IFN γ in the presence of a metalloproteinase inhibitor and could explain the proliferation decrease observed in the presence of BB-3103 since IFN γ inhibits Th₂ type proliferation (Oriss *et al.* 1997).

In order to explain how BB-3103 could differently affect Th₁ and Th₂ type cells, it was postulated that CD62L could be differentially expressed between those two cell types. This was investigated in murine CD4⁺ T cells skewed with exogenous cytokines and neutralising antibodies and revealed that Th₁ cells could maintain CD62L expression whilst Th₂ cells lost CD62L upon activation. This was also recently demonstrated (Wely *et al.* 1999) and from this study, it emerged that the presence of IL-12 was responsible for upregulating CD62L message in Th₁ cell types, although metalloproteinase activity was not investigated.

No IL-12 was detectable in the proliferation assays from human PBMCs, although the assay used in this assay may not have been sensitive enough. CD62L expression on human Th₁ and Th₂ cells and human Th₀ and Th₂ clones, unfortunately, was not investigated. There are, however to date, no reports suggesting that lymphocytes isolated from either atopic or non-atopic patients differentially express CD62L.

Since CD62L expression is maintained on activated CD4⁺ Th₁ cells, it would have been expected that inhibition of CD62L shedding (since CD62L is still present on the surface of the cell) would further inhibit cell proliferation by reducing IL-2 production.

To address this aspect further, however, it would have been interesting to investigate whether Th₁ and Th₂ cells are capable of re-expressing CD62L at different rates which would explain the differential expression at the cell surface. Alternatively, the upregulation of CD62L on Th₁ cells may be due to a combination of both mRNA upregulation (driven by IL-12 signalling (Wely 1999) and loss of metalloproteolytic activity, whereas Th₂ cells may maintain or increase metalloproteolytic activity.

In order for BB-3103 to interfere with proliferation when added at later time points (24 or 48hrs) to proliferation assays, the inhibitor would be expected to interfere with the IL-2 signalling pathway since T lymphocytes express IL-2 within a few hours of TCR mediated activation (Cantrell 1996). Studies with HT-2 cells, however, revealed that BB-3103 does not inhibit IL-2 driven signalling. These findings suggest there may be other unidentified metalloproteinase-sensitive molecules which are differentially expressed on Th₁ and Th₂ type cells.

CONCLUSIONS

The results shown in this thesis indicated that metalloproteinase inhibitors could, potentially, be useful in controlling or redressing deleterious Th₁ vs. Th₂ immune imbalances found in diseased states such as allergic asthma. Much more investigation, however, should be devoted into assessing the phenotypic and signalling differences between Th₁ and Th₂ cells in the presence of the inhibitor, since, the discrepancies in proliferation observed between the two groups in this study may not solely be due to differential CD62L expression but also to other metalloproteinase-sensitive cell surface molecules which may differentially expressed by these two cell types.

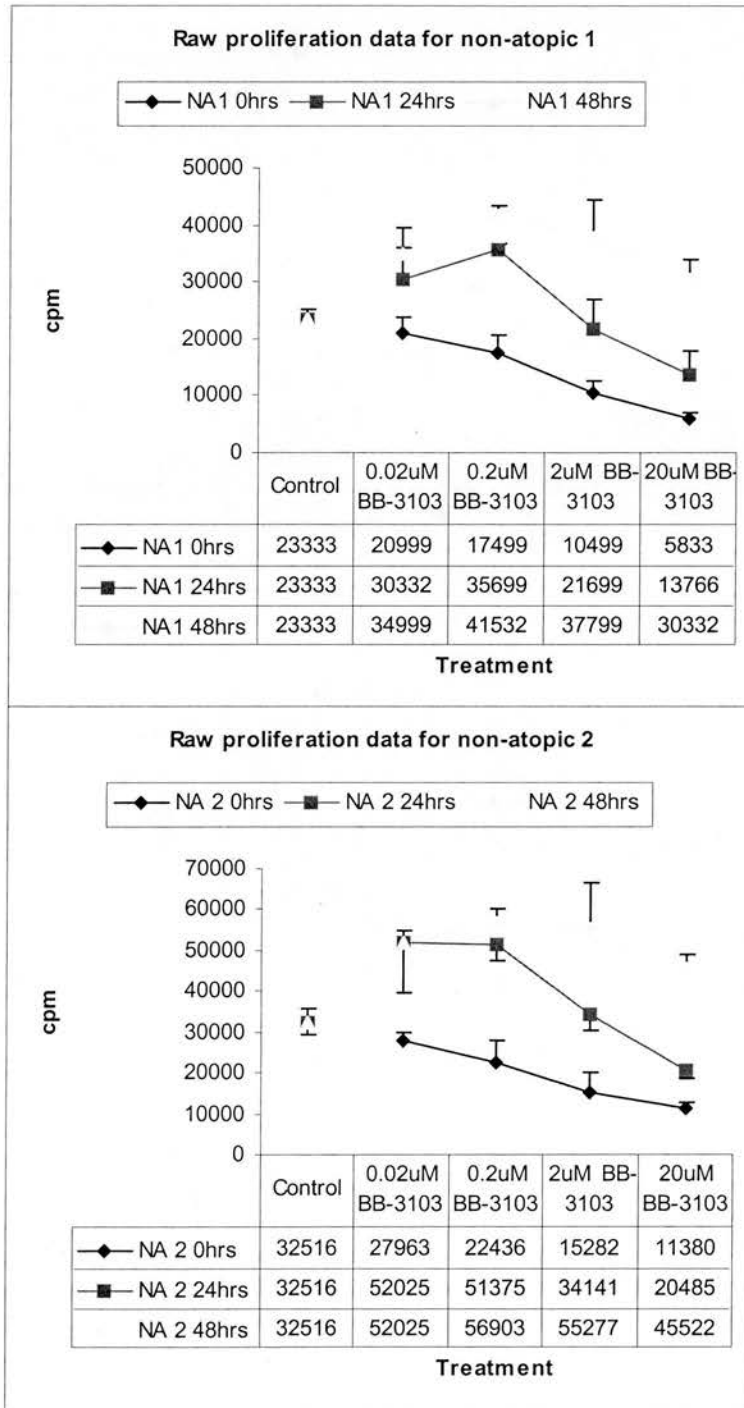
The majority of the work described in this thesis suggests that metalloproteinase activity is required for optimal activation of CD4⁺ T lymphocytes. The observed decrease in proliferation at certain time points of activated CD4⁺ T cells in the presence of a metalloproteinase inhibitor, BB-3103, was determined to be due to the inhibition of CD62L shedding, since the proliferation kinetics of CD62L gene deficient CD4⁺ T lymphocytes was unaffected by BB-3103. The results also indicate that CD62L is involved in regulation of signalling components upon CD4⁺ T cell activation, suggesting association of CD62L with signal transduction molecules involved in CD4⁺ T cell activation and subsequent IL-2 production. CD62L has previously been investigated as an important molecule involved in signal transduction in neutrophils. These results, however, are the first reports that CD62L is implicated in regulating T cell activation processes.

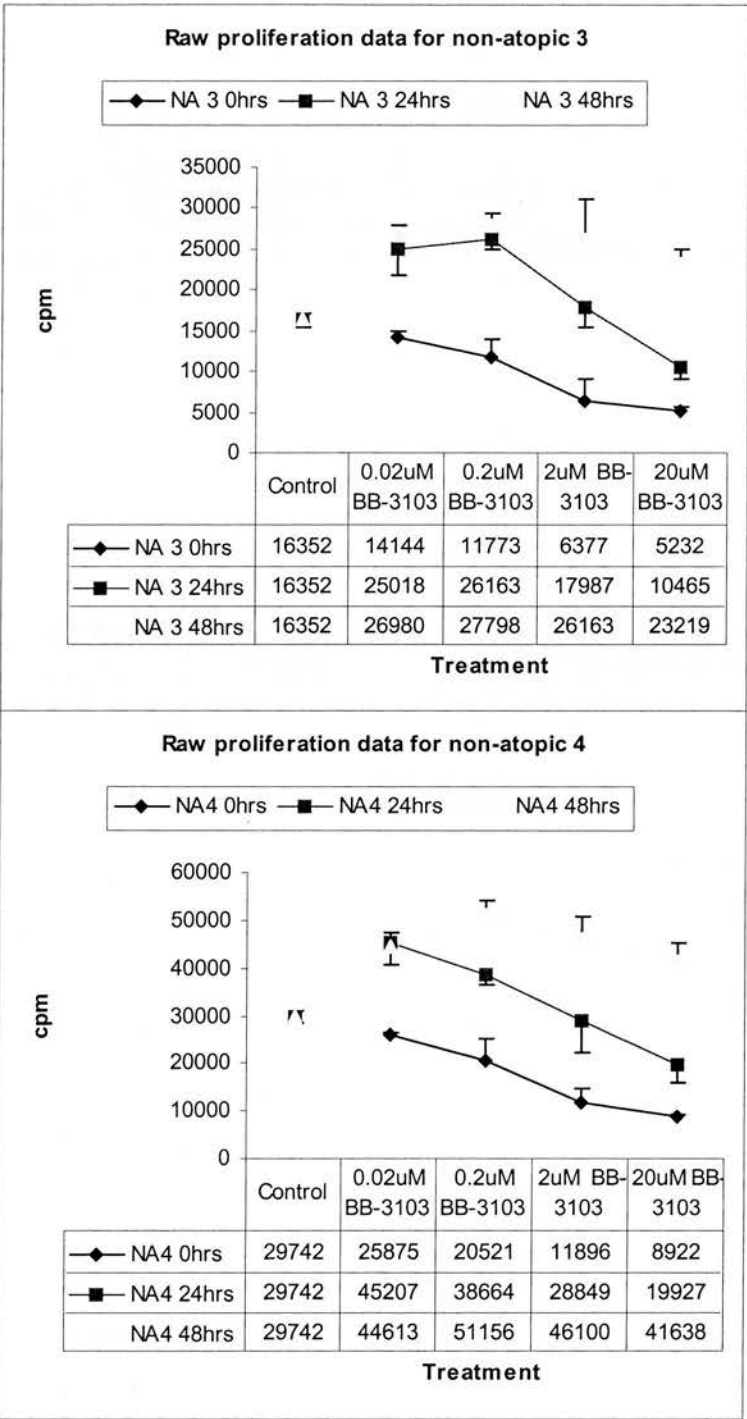
The presence of CD62L at the cell surface of naïve CD4⁺ T cells was originally thought only to mediate tethering of CD62L bearing cells to endothelial cells. It is now interesting, however, to consider that CD62L on CD4⁺ T lymphocytes may play a more important role, in

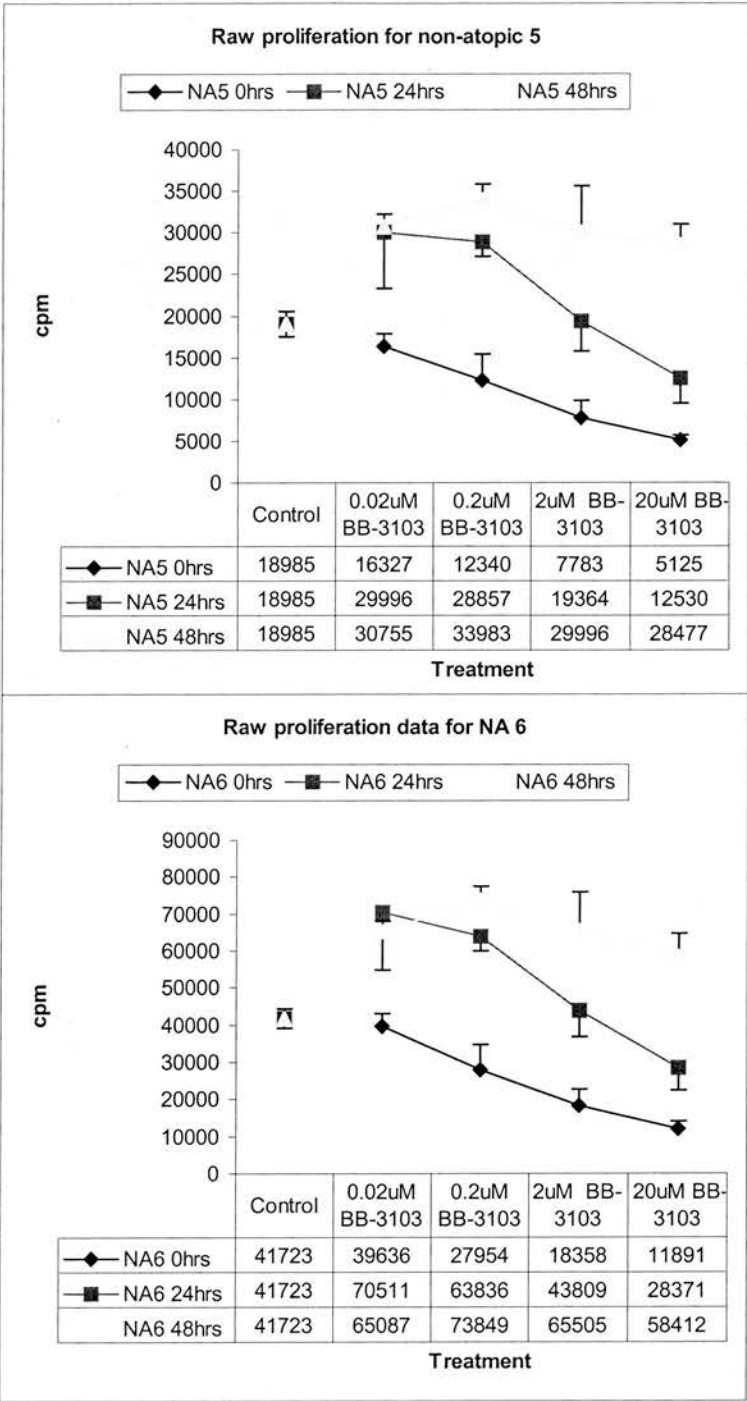
regulating the activation state of the cell through tyrosine kinases and calcium ions which lead to the expression of IL-2. Studies investigating the physical association of CD62L in naïve CD4⁺ T cells to intracellular signalling components involved in TCR mediated activation would have enabled further understanding of the role of CD62L in signal transduction. Since the general understanding in CD4⁺ T cell activation is now thought to involve conglomeration of lipid rich rafts containing transmembrane molecules and signal transducing proteins, it may be possible that CD62L is an integral member of the rafts which could regulate the amount of activation the CD4⁺ T cell undergoes.

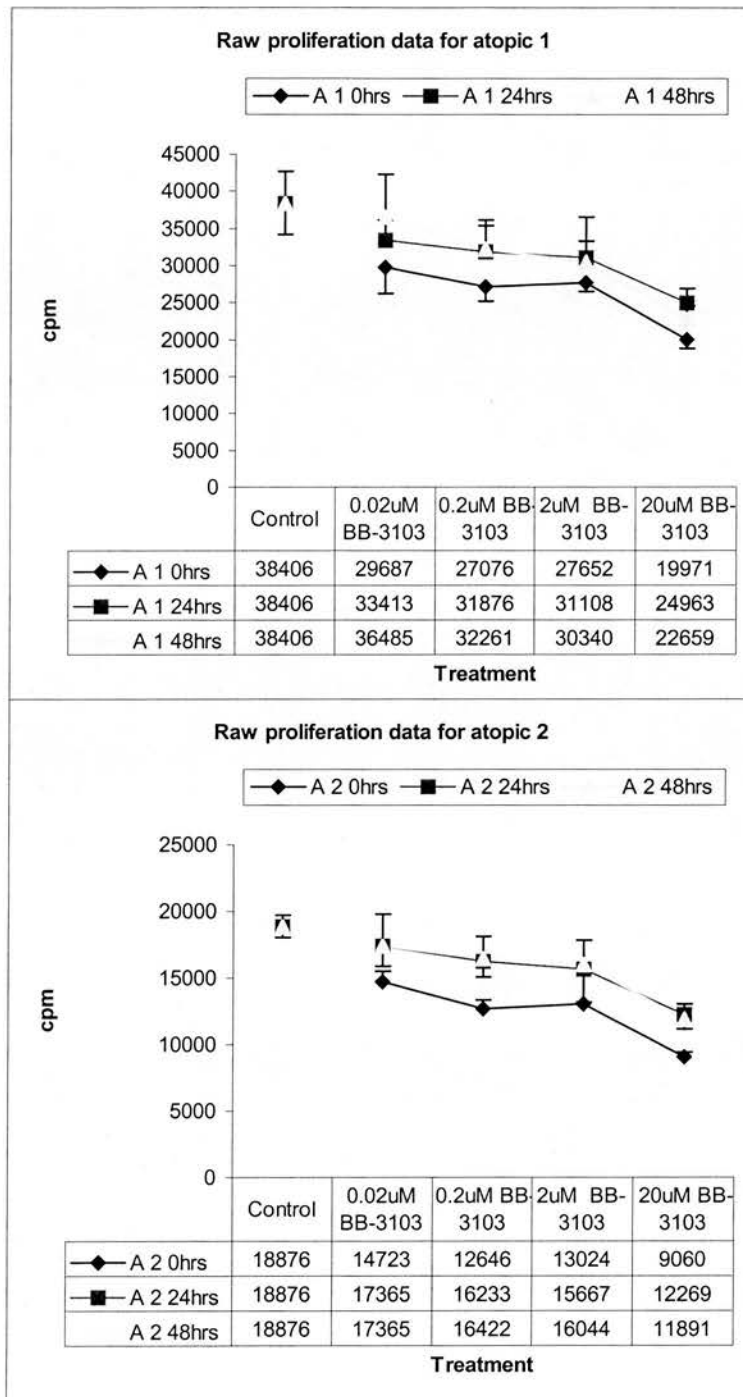
APPENDIX

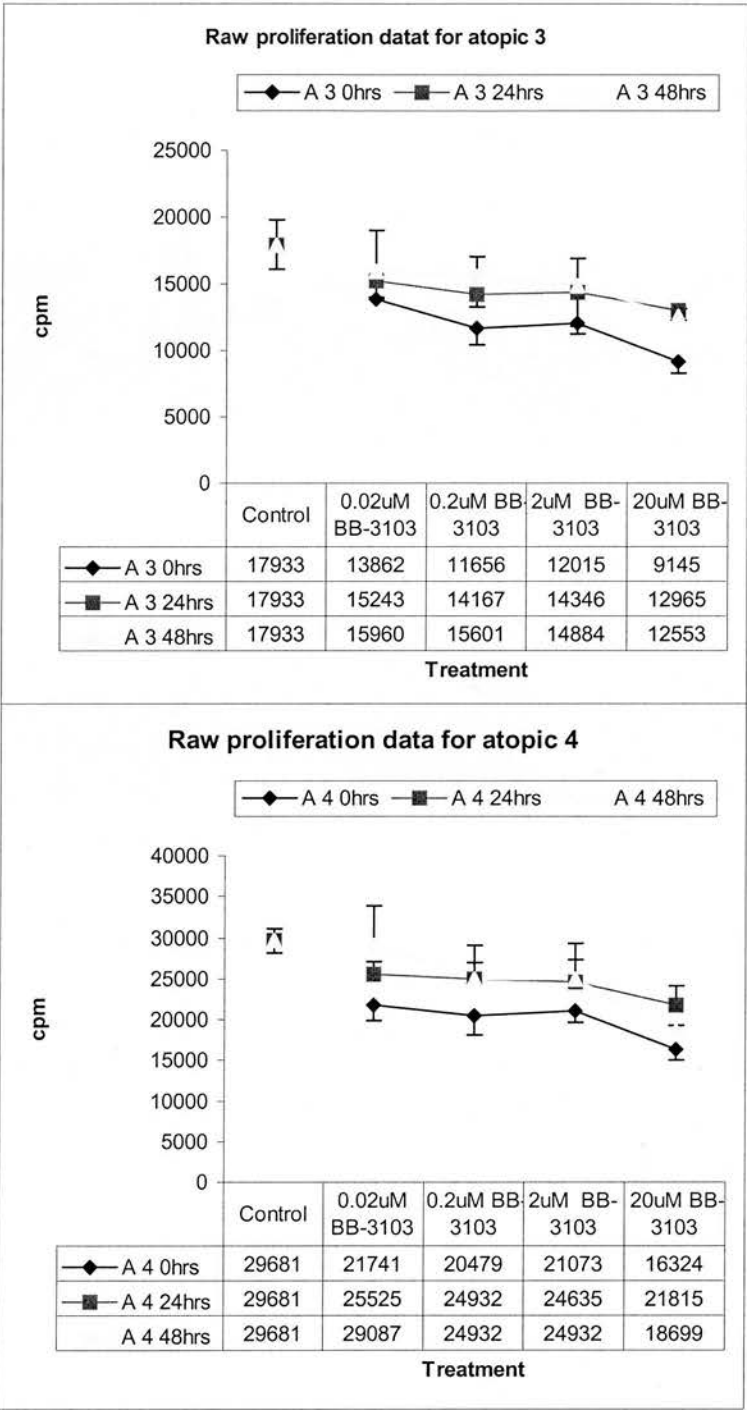
The following pages show the raw data obtained from PBMCs isolated from atopic and non-atopic individuals and stimulated with the aeroallergen Der p 1 isolated from *Dermatophagoides pteronyssinus* for 5 days as described in the materials and methods section.

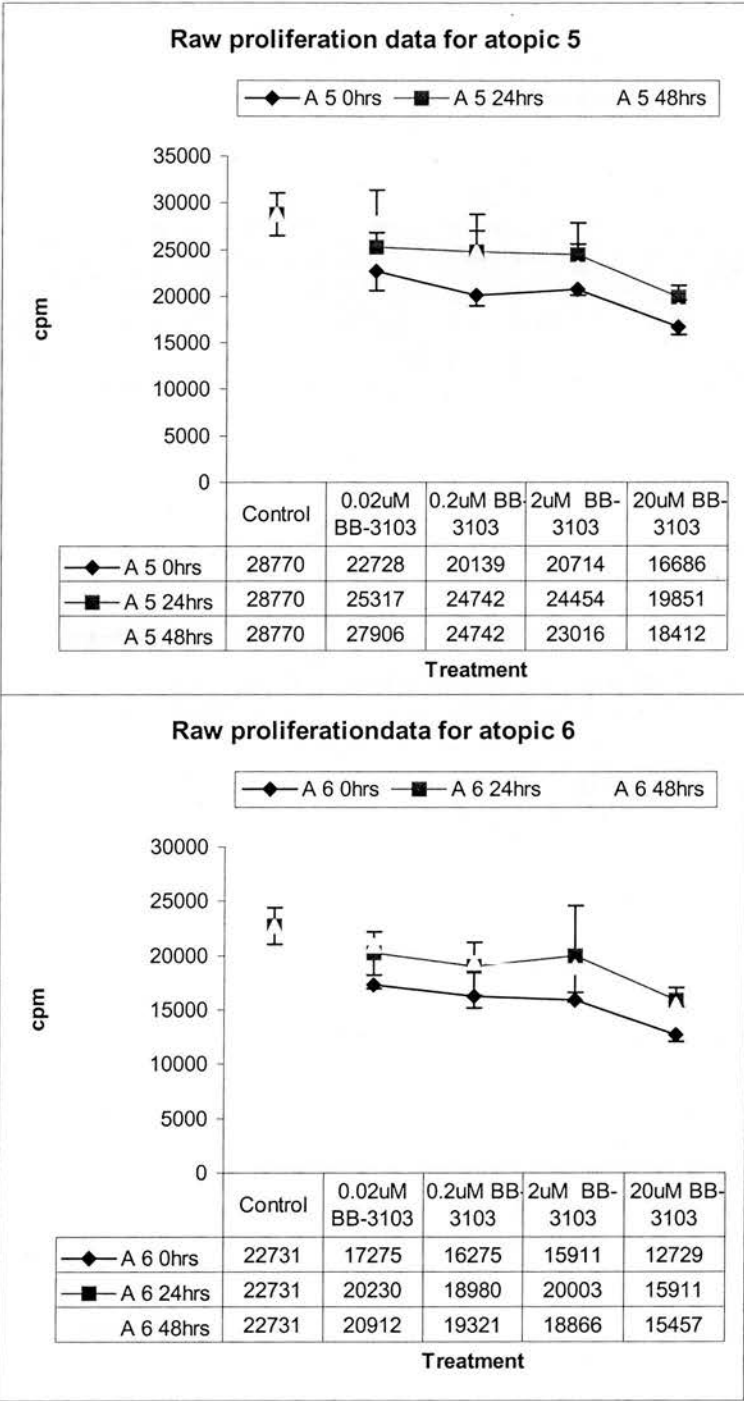












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